04-04-00

Customer Number: 000959

DIVISIONAL-CONTINUATION APPLICATION TRANSMITTAL FORM UNDER RULE 1.53(b) (former Rule 1.60)

DOCKET NUMBER	ANTICIPATED CLASSIFICATION OF THIS APPLICATION:		PRIOR APPLICATION SERIAL NUMBER: 08/456,864	PRIOR APPLICATION FILING DATE: 6/1/95
UIZ-003DVCN	CLASS:	SUBCLASS:	EXAMINER: P. BASKAR	ART UNIT: 1641

ASSISTANT COMMISSIONER FOR PATENTS
BOX PATENT APPLICATION
WASHINGTON, DC 20231

CERTIFICATION UNDER 37 CFR 1.10

Date of Deposit: April 3, 2000 Mailing Label Number: EL 263 574 916 US

I hereby certify that this 37 CFR 1.53(b) request and the documents referred to as attached therein are being deposited with the United States Postal Service on the date indicated above in an envelope as "Express Mail Post Office to Addressee" service under 37 CFR 1.10 and addressed to the Assistant Commissioner for Patents, Box Patent Application, Washington, D.C. 20231.

Viriato G. Cardoso — Name of Person Mailing Paper

Viriato G. Cardoso — Signature of Person Mailing Paper

Dear Sir:

This is a request for filing a ☒ continuation ☐ divisional application under 37 CFR 1.53(b), of pending prior application serial no. 08/456,864 filed on March 17, 1999, of Pearson *et al.* entitled AUTOINDUCER MOLECULE which in turn is a continued prosecution application of serial no. 08/456,864 filed on June 1, 1995, now abandoned, which in turn is a divisional application of serial no. 08/104,487 filed on August 9, 1993 and issued as U.S. Patent 5,591,872.

1. ☒ Enclosed is a copy of the latest inventor signed application, including the oath or declaration as originally filed. The copy of the enclosed papers is as follows:
   - ☒ 26 page(s) of specification
   - ☒ 6 page(s) of claims
   - ☒ 1 page(s) of abstract
   - ☒ 4 sheet(s) of informal drawings
   - ☒ 24 page(s) of executed declaration and power of attorney.

   I hereby verify that the attached papers are a true copy of the prior complete application serial no. 08/456,864 as originally filed on June 1, 1995.

2. ☒ 3 verified statements to establish small entity status under 37 CFR 1.9 and 1.27, copies of which are enclosed, were filed in the prior application and such status is still proper and desired (37 CFR 1.28(a)).

3. ☒ The filing fee is calculated below:

	NUMBER OF CLAIMS FILED			NUMBER EXTRA
TOTAL	* 1	MINUS	** 20	= 0
INDEP.	* 1	MINUS	*** 3	= 0
☐ MULTIPLE DEPENDENT CLAIMS				

SMALL ENTITY		OR	OTHER THAN A SMALL ENTITY	
RATE	FEE		RATE	FEE
x 9 =	$0.00		x 18 =	$.00
x 39 =	$0 00		x 78 =	$ 00
+130 =	$ 00		+ 260 =	$.00
BASIC FEE	$345 00		BASIC FEE	$ 00
TOTAL	$345 00	OR	TOTAL	$0 00

4. ☐ The Commissioner is hereby authorized to charge any additional fees which may be required in connection with this communication, or credit any overpayment, to Deposit Account No. 12-0080. A duplicate copy of this sheet is enclosed.

5. ☒ **The filing fee is not being paid at this time.**

6. ☒ Cancel in this application original claims 2-43 of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

7. ☐ A preliminary amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning with the number next following the highest numbered original claims in the prior application.)

8. ☒ Amend the specification by inserting before the first line the sentences: "This application is a continuation application of serial no. 08/456,864 filed on 3/17/99, pending, which in turn is a continued prosecution application of 08/456,864 filed on 6/1/95, now abandoned, which in turn is a divisional application of serial no. 08/104,487 filed on August 9, 1993 and issued as U.S. Patent 5,591,872. The contents of all of the aforementioned application(s) are hereby incorporated by reference."

9. ☐ Please abandon said prior application as of the filing date accorded this application. A duplicate copy of this transmittal is enclosed for filing in the prior application file. (May be used if signed by person authorized by §1.138 and before payment of base issue fee.)

10. ☒ Copy of formal drawings from parent application is enclosed (2 pages).

11. ☐ Priority of application serial no. _____ filed on _____ in _____ is claimed under 35 U.S.C. §119.
    - ☐ The certified copy has been filed in prior application serial no. _____ filed on _____.
    - ☐ The certified copy will follow.

12. ☒ The prior application is assigned of record to the University of Iowa, the University of Rochester and Ithaca College.

13. ☐ A _____ month extension of time has been submitted in the parent application Serial No. in order to establish copendency with the present application.

14. ☒ Also enclosed is an executed Associate Power of Attorney.

15. ☐ The power of attorney in the prior application is to Lahive & Cockfield, LLP.

    a. ☒ The power appears in the original papers in the prior application, copies attached.

    b. ☐ Since the power does not appear in the original papers, a copy of the power in the prior application is enclosed.

    c. ☐ A new power has been executed and is attached.

16. ☒ Address all future communications (May only be completed by applicant, or attorney or agent of record) to Elizabeth A. Hanley, Esq. at **Customer Number: 000959** whose address is:

Lahive & Cockfield, LLP
28 State Street
Boston, Massachusetts 02109

17. ☒ Any requests for extensions of time necessary in a parent application for establishing copendency between this application and a parent application are hereby requested and the Commissioner is authorized to charge any fee associated with such an extension to Deposit Account No. 12-0080.

18. ☐ Pursuant to 37 CFR 1.821(e), the computer readable form of the sequence listing for this new application is to be identical with the computer readable form of application serial no._____. Please use the computer readable form of application serial no._____ in lieu of filing a duplicate computer readable form in this application. Pursuant to 37 CFR 1.821(f), the content of the paper copy of the sequence listing for this new application and the computer readable form of application serial no._____ are the same.

April 3, 2000
Date

Peter C. Lauro, Esq.
Reg. No. 32,360
☐ inventor(s) ☐ filed under §1.34(a)
☐ assignee of complete interest
☒ attorney or agent of record

LAHIVE & COCKFIELD, LLP
28 State Street
Boston, Massachusetts 02109
Tel. (617) 227-7400

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: James P. Pearson, et al.

Serial No.: 104,487

Filed: August 9, 1993

Title: AN AUTOINDUCER MOLECULE

Attorney Docket No.: UIZ-003

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) & 1.27(d))--NONPROFIT ORGANIZATION

I HEREBY DECLARE THAT I AM AN OFFICIAL EMPOWERED TO ACT ON BEHALF OF THE NONPROFIT ORGANIZATION IDENTIFIED BELOW:

NAME OF NONPROFIT ORGANIZATION: THE UNIVERSITY OF IOWA RESEARCH FOUNDATION

ADDRESS OF NONPROFIT ORGANIZATION: 214 TECHNOLOGY INNOVATION CENTER, OAKDALE RESEARCH CAMPUS, IOWA CITY, IOWA 52319

TYPE OF NONPROFIT ORGANIZATION

- ☐ UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
- ☐ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 U.S.C. 501(a) and 501(c)(3))
- ☒ NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
  (NAME OF STATE Iowa
  (CITATION OF STATUTE CHAPTER 504A, CODE OF IOWA

I HEREBY DECLARE THAT THE NONPROFIT ORGANIZATION IDENTIFIED ABOVE QUALIFIES AS A NONPROFIT ORGANIZATION AS DEFINED IN 37 CFR 1.9(E) FOR PURPOSES OF PAYING REDUCED FEES TO THE UNITED STATES PATENT AND TRADEMARK OFFICE REGARDING THE INVENTION ENTITLED: AN AUTOINDUCER MOLECULE BY INVENTOR(S) JAMES P. PEARSON, ET AL.

DESCRIBED IN:

- ☐ THE SPECIFICATION FILED HEREWITH.
- ☒ APPLICATION SERIAL NO. 104,487 FILED: August 9, 1993
- ☐ PATENT NO. _____, ISSUED _____

I HEREBY DECLARE THAT RIGHTS UNDER CONTRACT OR LAW HAVE BEEN CONVEYED TO AND REMAIN WITH THE NONPROFIT ORGANIZATION REGARDING THE ABOVE-IDENTIFIED INVENTION.

IF THE RIGHTS HELD BY THE NONPROFIT ORGANIZATION ARE NOT EXCLUSIVE, EACH INDIVIDUAL, CONCERN OR ORGANIZATION HAVING RIGHTS IN THE INVENTION IS LISTED BELOW* AND NO RIGHTS TO THE INVENTION ARE HELD BY ANY PERSON, OTHER THAN THE INVENTOR WHO WOULD NOT QUALIFY AS AN INDEPENDENT INVENTOR UNDER 37 CFR 1.9(C) IF THAT PERSON MADE THE INVENTION, OR BY ANY CONCERN WHICH WOULD NOT QUALIFY AS A SMALL BUSINESS CONCERN UNDER 37 CFR 1.9(D) OR A NONPROFIT ORGANIZATION UNDER 37 CFR 1.9(E).

*NOTE: SEPARATE VERIFIED STATEMENTS ARE REQUIRED FROM EACH NAMED PERSON, CONCERN OR ORGANIZATION HAVING RIGHTS TO THE INVENTION AVERRING TO THEIR STATUS AS SMALL ENTITIES (37 CFR 1.27)

NAME Ithaca College
ADDRESS Ithaca, New York 14850
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☒ NONPROFIT ORGANIZATION

NAME University Of Rochester
ADDRESS Rochester, New York 14627
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☒ NONPROFIT ORGANIZATION

I ACKNOWLEDGE THE DUTY TO FILE, IN THIS APPLICATION OR PATENT, NOTIFICATION OF ANY CHANGE IN STATUS RESULTING IN LOSS OF ENTITLEMENT TO SMALL ENTITY STATUS PRIOR TO PAYING, OR AT THE TIME OF PAYING, THE EARLIEST OF THE ISSUE FEE OR ANY MAINTENANCE FEE DUE AFTER THE DATE ON WHICH STATUS AS A SMALL ENTITY IS NO LONGER APPROPRIATE. (37 CFR 1.28(B))

I HEREBY DECLARE THAT ALL STATEMENTS MADE HEREIN OF MY OWN KNOWLEDGE ARE TRUE AND THAT ALL STATEMENTS MADE ON INFORMATION AND BELIEF ARE BELIEVED TO BE TRUE; AND FURTHER THAT THESE STATEMENTS WERE MADE WITH THE KNOWLEDGE THAT WILLFUL FALSE STATEMENTS AND THE LIKE SO MADE ARE PUNISHABLE BY FINE OR IMPRISONMENT, OR BOTH, UNDER SECTION 1001 OF TITLE 18 OF THE UNITED STATES CODE, AND THAT SUCH WILLFUL FALSE STATEMENTS MAY JEOPARDIZE THE VALIDITY OF THE APPLICATION, ANY PATENT ISSUING THEREON, OR ANY PATENT TO WHICH THIS VERIFIED STATEMENT IS DIRECTED.

NAME OF PERSON SIGNING W. BRUCE WHEATON

TITLE IN ORGANIZATION OF PERSON SIGNING Executive Director, The University of Iowa Research Foundation

ADDRESS OF PERSON SIGNING 214 Technology Innovation Center, Oakdale Research Campus, Iowa City, Iowa 52319

SIGNATURE _____ DATE 9/24/93

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: James P. Pearson, et al.
Serial No.: 104,487
Filed: August 9, 1993
Title: AN AUTOINDUCER MOLECULE

Attorney Docket No.: UIZ-003

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NAME OF NONPROFIT ORGANIZATION UNIVERSITY OF ROCHESTER

ADDRESS OF NONPROFIT ORGANIZATION ROCHESTER, NEW YORK 14627

ADDRESS OF NONPROFIT ORGANIZATION
- ☒ UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
- ☐ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 U.S.C. 501(a) and 501(c)(3))
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(CITATION OF STATUTE

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DESCRIBED IN:
- ☐ THE SPECIFICATION FILED HEREWITH.
- ☒ APPLICATION SERIAL NO. 104,487 FILED: August 9, 1993
- ☐ PATENT NO._____, ISSUED_____

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NAME The University of Iowa Research Foundation
ADDRESS 214 Technology Center, Oakdale Research Campus, Iowa City, Iowa
- ☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☒ NONPROFIT ORGANIZATION

NAME Ithaca College
ADDRESS Ithaca, New York 14627
- ☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☒ NONPROFIT ORGANIZATION

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NAME OF PERSON SIGNING JANE YOUNGERS

TITLE IN ORGANIZATION OF PERSON SIGNING Director - Office of Research, University of Rochester

ADDRESS OF PERSON SIGNING 518 Hylan Building, Rochester, New York 14627

SIGNATURE Jane A Youngers DATE 29 September 1993

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: James P. Pearson, et al.
Serial No.: 104,487
Filed: August 9, 1993
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Attorney Docket No.: UIZ-003

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NAME The University of Iowa Research Foundation
ADDRESS 214 Technology Innovation Center, Oakdale Research Campus, Iowa City, Iowa 52319
- ☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☒ NONPROFIT ORGANIZATION

NAME University Of Rochester
ADDRESS Rochester, New York 14627
- ☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☒ NONPROFIT ORGANIZATION

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NAME OF PERSON SIGNING MARGARET BALL

TITLE IN ORGANIZATION OF PERSON SIGNING Vice President/College Counsel, Ithaca College,

ADDRESS OF PERSON SIGNING Job Hall, Ithaca, New York 14850

SIGNATURE Margaret T. Ball DATE 10/1/93

AN AUTOINDUCER MOLECULE

Government Support

This research was supported by grants and fellowships from the Cystic Fibrosis Foundation, Office of Naval Research (N00014-80-6570), National Institute of Allergy and Infectious Diseases (33713), and National Science Foundation (DIR9017262).

Background

The Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic human pathogen that causes infections in immunocompromised hosts, and colonizes the lungs of individuals with cystic fibrosis (Hoiby, N. (1974) *Acta Patholgica Microbiol. Scand.* Sect. B. 82, 551-558; Reynolds, H.Y., Levine, A.S., Wood, R.E., Zierdt, C.H., Dale, D.C. and Pennington, J.L. (1975) *Ann. Intern. Med.* 82, 819-832). This bacterium produces a number of extracellular virulence factors including exotoxin A, which is encoded by the *toxA* gene (Iglewski, B.H. and Kabat, D. (1975) *Proc. Natl. Acad. Sci. USA.* 72, 22842288; Iglewski, B.H., Sadoff, J.C., Bjorn, M.J., and Maxwell, E.S. (1978) *Proc. Natl. Acad. Sci. USA.* 75, 3211-3215), two elastolytic proteases, encoded by the *lasA* and *lasB* genes, and an alkaline protease encoded by the *aprA* gene (Morihara, K. and Homma, J.Y. (1985) in *Bacterial Enzymes and Virulence*, ed. Holder, I.A. (CRC Press, Boca Raton, Florida) pp. 41-79; Bever, R.A. and Iglewski, B.H. (1988) *J. Bacteriol.* 170, 4309-4313; Kessler, E. and Saffrin, M. (1988) *J. Bacteriol.* 170, 5241-5247).

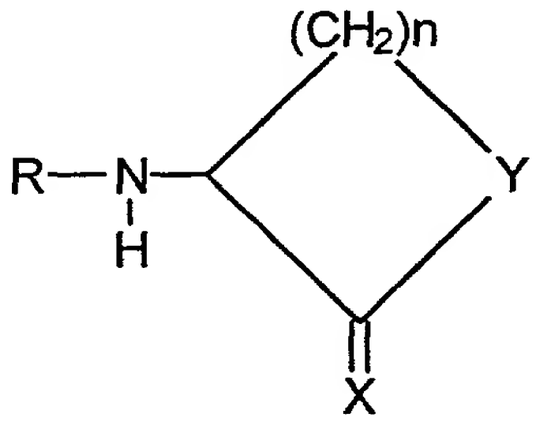
Autoinducer molecules are capable of regulating the gene expression of certain microorganisms. Bycroft et al. (WO92/18614) describe a class of autoinducer molecules which includes N-(β-ketocaproyl) L-homoserine lactone and N-(β-hydroxybutyryl) homoserine lactone. All of the exemplified autoinducer molecules of Bycroft et al. contain C_1-C_7 side chains. However, autoinducer molecules with side chains of greater length or cyclic side chains are not exemplified by Bycroft et al..

Bycroft et al. state that *Pseudomonas aeruginosa* is affected by N-(β-ketocaproyl) homoserine lactone. As recently as 1993, researchers have believed that N-(β-ketocaproyl) homoserine lactone is the autoinducer

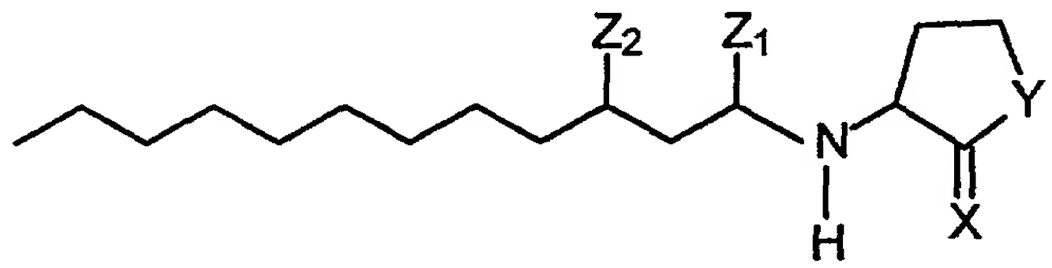
molecule of *P. aeruginosa*. (Stewart, G.S.A.B. and P. Williams (1993) *ASM News*, **59**, 241-46)

Summary of the Invention

The present invention is based, in least in part, on the discovery that the autoinducer molecule for *Pseudomonas aeruginosa* is N-(3-oxododecanoyl)homoserine lactone and not N-(β-ketocaproyl)homoserine lactone as previously believed. Upon the discovery of this novel autoinducer molecule, it was realized that autoinducer molecule(s) containing a fatty moiety or a moiety having at least seven members in the R moiety of the formula set forth below:



are involved in the regulation of gene expression. In the above formula, n is 2 or 3; Y is O, S, or NH; X is O, S, or NH; and R is a fatty hydrocarbon or acyl moiety that may be substituted or a moiety having at least seven members containing a ring structure that may be substituted. The present invention further pertains to autinducer molecules of the following formula:



wherein X and Y are as defined above and Z_1 and Z_2 are independently selected from the group consisting of hydrogen, **=O**, **=S**, and **=NH**.

The present invention also pertains to analogs of the autoinducer molecule that affect the activity of the LasR protein, e.g., inhibit the autoinducer activity or synergistically enhance the autoinducer activity. The present invention even further includes inhibitors of the autoinducer

activity of N-(3-oxododecanoyl)homoserine lactone. The present invention also pertains to methods of selecting such inhibitors and analogs. These methods involve the contact of the autoinducer molecule with the suspected inhibitor or synergist followed by the measuring of the ability of the treated autoinducer molecule to perform its intended function. From these steps, it is determined whether the suspected inhibitor or synergist inhibits or enhances the ability of the autoinducer molecule to stimulate the activity of the selected gene.

The present invention also pertains to therapeutic compositions comprising an agent having the ability to inhibit the activity of the LasR protein of *Pseudomonas aeruginosa* and/or inhibit the autoinducer activity of N-(3-oxododecanoyl)homoserine lactone and a pharmaceutically acceptable carrier. The agent can be the analogs or inhibitors as described above and in further detail below.

The present invention even further pertains to a method of inhibiting the infectivity of *Pseudomonas aeruginosa* and a method of treating an immunocompromised individual infected with *Pseudomonas aeruginosa*. Both of these methods involve the administration to an individual of a therapeutically effective amount of the agents and/or therapeutic compositions described above that inhibit the activity of the LasR protein and/or inhibit the autoinducer activity of N-(3-oxododecanoyl)homoserine lactone. An example of an immunocompromised individual in an individual afflicted with cystic fibrosis.

The present invention further pertains to a culture medium containing as an added compound an autoinducer molecule as described and methods of controlling the expression of a gene in bacteria or cells using the described autoinducer molecules and/or analogs or inhibitors thereof.

The present invention also pertains to analogs of the autoinducer molecule that inhibit the induction of virulence factors by the autoinducer molecule or LasR. The virulence factors include exotoxin A, elastolytic proteases, and an alkaline protease.

Brief Description of the Figures

Figure 1 shows the dose-response curve for *P. aeruginosa* autoinducer in ethyl acetate extracts of bacterial culture fluid. Culture fluid extracts were of *P. aeruginosa* PA01 (•), *P. aeruginosa* PAO1-Rl (○), *E. coli* with pLasI-l (■), and *E. coli* without pLasI-l (□). As defined in Materials and Methods, a unit of autoinducer activity is that amount required for half-maximal activation of the *lasB* promoter in *E. coli* (pKDT17).

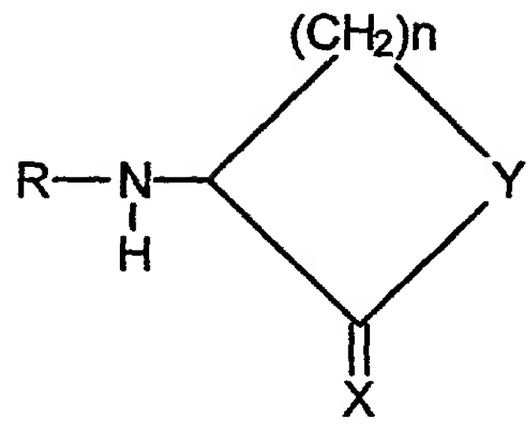
Figure 2 shows the HPLC analysis of autoinducer extracts. Extracted autoinducer was from an *E. coli* (pLasI-l) culture medium (○), and from a *P. aeruginosa* PA01 culture medium (•). Methanol-gradient HPLC was as described in Materials and Methods. Each fraction was 2 ml. The dashed line indicates the methanol concentration. The percent of activity recovered in the major peak for either bacterium was >75%. The white triangle indicates where N-(3-oxohexanoyl)homoserine lactone elutes, and the black triangle indicates where N-(3-oxo-octanoyl)homoserine lactone elutes.

Figure 3 shows the dose-response curve for activity of synthetic N-(3-oxododecanoyl)homoserine lactone in the *P. aeruginosa* autoinducer bioassay.

Figure 4 shows the known autoinducer structures. From the top, N-(3-hydroxybutanoyl)homoserine lactone, the inducer of luminescence in *Vibrio harveyi;* N(3-oxohexanoyl)homoserine lactone, the inducer of *V. fischeri* luminescence; N-(3oxooctanoyl)homoserine lactone, the inducer of conjugal transfer genes in *Agrobacterium tumefaciens*; and N-(3-oxododecanoyl)homoserine lactone, the *P. aeruginosa* autoinducer of the present invention.

Detailed Description

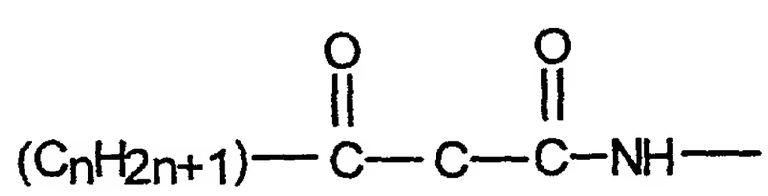
The present invention pertains to autoinducer molecules of the formula:



wherein n is 2 or 3; Y is O, S, or NH; X is O, S, or NH; and R is a fatty hydrocarbon or acyl moiety that may be substituted or a moiety having at least seven members containing a ring structure that may be substituted. The autoinducer molecule regulates the activity of the LasR protein of *Pseudomonas aeruginosa*. In addition, the present invention pertains to optically active isomers of the autoinducer molecule. The autoinducer molecule can be purified from the native source using conventional techniques or can be derived synthetically by chemical means. Included in the invention are optically active isomers of the claimed autoinducer molecule as well as analogs of the claimed autoinducer molecule.

The language "autoinducer molecule" is intended to include a molecule involved in the regulation of gene expression, e.g., it may increase or decrease gene expression, of a microorganism. Typically, autoinducer molecules are produced by microorganisms, such as bacteria, during metabolism. The autoinducer molecules then regulate gene expression, for example, by combining with a transcriptional activator protein.

The language "fatty hydrocarbon or acyl moiety" is intended to include a long straight or branched chain moiety having seven or more carbon atoms. For example, the fatty acyl moiety can be of the following formula:



wherein n is 4 or more. The preferred fatty acyl moieties include C_7 - C_{14} acyl moieties, more preferred are C_{10} - C_{14} acyl moieties, and most preferred is the C_{12} acyl moiety. Fatty hydrocarbon or acyl moieties include saturated and unsaturated moieties as well as substituted moieties, for example, by

substituting a S for an O. Examples of fatty hydrocarbon moieties include fatty alkyl, fatty alkenyl, and fatty alkynyl moieties.

The language "ring structure" is intended to include arrangements of atoms which form one or more rings. The ring structures can contain heterocyclic ring(s), e.g., oxygen, sulfur, or nitrogen containing, or can contain carbocyclic ring(s). The ring structure further can be a fused ring system. Examples of ring structures include 5 to 7 membered heterocyclic rings, napthyl, and phenyl. The ring structures further can be substituted with groups that do not effect the molecule's ability to perform its intended function as described above.

The substituents on the "R" moiety are substituents which do not detrimentally effect the molecule's ability to perform its intended autoinducer function. Examples of such substituents include hydrocarbon groups, e.g., lower alkyl, alkenyl and alkynyl groups, keto groups and halogen containing substituents. Examples of such substituents include butyl, propyl, methyl, butenyl, propenyl and butynyl groups.

The language "able to regulate the activity" is intended to include the activation of or an act to increase the operation of another molecule, e.g., the LasR protein.

The language "LasR protein of *Pseudomonas aeruginosa*" is intended to include the transcriptional activator proteins of the bacteria *Pseudomonas aeruginosa*. LasR proteins of *P. aeruginosa* include proteins encoded by the *lasR* gene of *P. aeruginosa*. The LasR proteins are global regulators of genes involved in the virulence of *P. aeruginosa*.

The language "isomer" is intended to include molecules having the same molecular formula as the autoinducer molecule but possessing different chemical and physical properties due to a different arrangement of the atoms in the molecule. Isomers include both optical isomers and structural isomers.

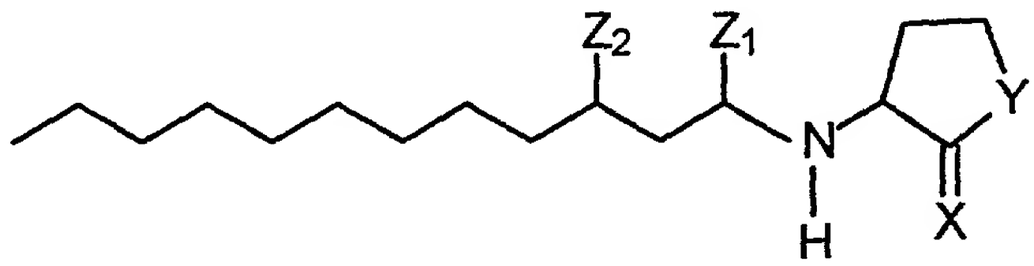
The language "optically active" is intended to include molecules that have the ability to rotate a plane of polarized light. An optically active isomer includes the L-isomer and the D-isomer of the claimed autoinducer molecule. The L-isomer of N-(3-oxododecanoyl)homoserine lactone is the active form. The D-isomer shows a small amount of activity

and can inhibit the ability of the L-isomer to activate the LasR protein by attaching to the autoinducer binding domain of the LasR protein.

The language "purified from the native source" is intended to include an autoinducer molecule of the above formula that has been manufactured by an organism. "Purified from the native source" includes isolating the autoinducer molecule from the culture media or cytoplasm of bacteria such as *Pseudomonas aeruginosa* using conventional techniques.

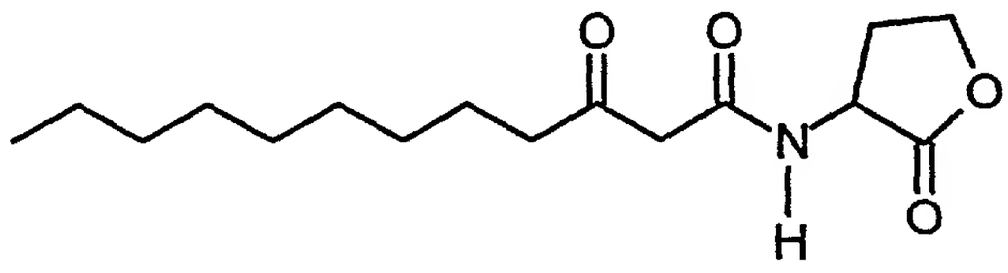
The language "synthesized by chemical means" is intended to include autoinducer molecules of the claimed formula that have been made artificially outside of an organism. The invention includes the claimed autoinducer made by a scientist in a laboratory from chemical precursors using standard chemical synthesis techniques. For example, the claimed autoinducer molecules can be synthesized using the protocol of Eberhard et al. (Eberhard, A., Burlingame, A. L., Eberhard, C., Kenyon, G. L., Nealson, K. H. and Oppenheimer, N. J. (1981) *Biochemistry* 20, 2444-2449) from commercially available precursors. The starting materials can be modified to produce the desired end product.

The present invention further pertains to autinducer molecules of the following formula:



wherein X and Y are as defined above and Z_1 and Z_2 are independently selected from the group consisting of hydrogen, **=O**, **=S**, and **=NH**.

The preferred autoinducer molecule of the present invention is of the formula:



This autoinducer molecule is a novel chemical compound which is at least part of the present invention. The new chemical compound has utility as an autoinducer and also may have other utilities. As an autoinducer, it can regulate the activity of the transcriptional protein of *Pseudomonas aeruginosa*, LasR. The chemical name of the autoinducer molecule is N-(3-oxododecanoyl)homoserine lactone. The autoinducer molecule can be purified from the native source or can be derived synthetically by chemical means. Included in the invention are optically active isomers of the claimed autoinducer molecule as well as analogs of the claimed autoinducer molecule.

The language "analog" is intended to include molecules that are structurally similar but not identical to the claimed autoinducer molecule N-(3-oxododecanoyl)homoserine lactone. For example, the length of the fatty acyl moiety can be varied producing an analog or one of the keto groups can be removed from the fatty acyl moiety. Analogs include autoinducer molecules that are structurally similar to the claimed autoinducer molecule but can inhibit rather than stimulate the activity of the LasR protein or analogs which act synergistically to enhance the ability of the claimed autoinducer to increase the activity of the LasR protein. One of ordinary skill in the art would be able to select analogs which are useful within the present invention using the selection methods described below.

The present invention also pertains to methods of selecting inhibitors or synergists of the autoinducer molecule, N-(3-oxododecanoyl)homoserine lactone. The method comprises contacting the autoinducer molecule with a suspected inhibitor or synergist, measuring the ability of the treated autoinducer molecule to stimulate the activity of a selected gene then determining whether the suspected inhibitor or synergist represses or enhances the activity of the autoinducer molecule. Actual inhibitors and synergists of the autoinducer molecule are then selected. For example, a suspected inhibitor can be mixed with N-(3-oxododecanoyl)homoserine lactone and the mixture then combined with *E. coli* MG4 which produces β-galactosidase in the presence of N-(3-oxododecanoyl)homoserine lactone. The amount of β-galactosidase can then be compared to a standard to determine if the suspected inhibitor represses

the ability of N-(3-oxododecanoyl)homoserine lactone to stimulate the production of β-galactosidase in *E. coli* MG4.

The language "inhibitors of the autoinducer molecule of *P. aeruginosa*" is intended to include molecules that interfere with the ability of the autoinducer molecule to stimulate the activity of the LasR protein of *P. aeruginosa*. Inhibitors include molecules that degrade or bind to N-(3-oxododecanoyl)homoserine lactone. The inhibitors can compete with the autoinducer molecule not allowing it to perform its intended function.

The language "synergist of the autoinducer molecule of *P. aeruginosa*" is intended to include molecules that enhance the ability of the autoinducer molecule to stimulate the LasR protein. Synergists include molecules that bind to either N-(3-oxododecanoyl)homoserine lactone or the LasR protein.

The present invention also pertains to methods of selecting inhibitory and synergistic analogs of the claimed autoinducer. The method comprises mixing a known amount of the autoinducer molecule with a known amount of the suspected inhibitory or synergistic analog, measuring the ability of the treated autoinducer molecule to stimulate the activity of a selected gene then determining whether the suspected inhibitory or synergistic analog represses or enhances the activity of the autoinducer molecule. Actual inhibitory or synergistic analogs of the autoinducer molecule are then selected.

The present invention further pertains to methods of inhibiting the infectivity of *P. aeruginosa*, methods for treating an immunocompromised host infected by *P. aeruginosa*, e.g., a person afflicted with cystic fibrosis, as well as therapeutic compositions. The methods comprise administering to an individual a therapeutically effective amount of an agent that is capable of inhibiting the activity of the LasR protein.

The language "inhibiting the infectivity of *P. aeruginosa*" is intended to include methods of affecting the ability of *P. aeruginosa* to initially infect or further infect an organism. This includes using agents that prevent the LasR protein from activating the transcription of extracellular virulence factors such as exotoxin A and elastolytic proteases by *P. aeruginosa*.

The language "agent" is intended to include molecules that inhibit the ability of the LasR protein to activate transcription of extracellular virulence factors. Agents include inhibitors of N-(3-oxododecanoyl)homoserine lactone. Agents also include analogs of N-(3-oxododecanoyl)homoserine lactone that can directly inhibit the LasR protein of *P. aeruginosa* or can compete with N-(3-oxododecanoyl)homoserine lactone. Inhibitory agents can be selected using the method described above.

The language "administering a therapeutically effective amount" is intended to include methods of giving or applying an agent to an organism which allow the agent to perform its intended therapeutic function. The therapeutically effective amounts of the agent will vary according to factors such as the degree of infection in the individual, the age, sex, and weight of the individual, and the ability of the agent to inhibit the activity of the LasR protein of *P. aeruginosa* in the individual. Dosage regima can be adjusted to provide the optimum therapeutic response. For example, several divided doses can be administered daily or the dose can be proportionally reduced as indicated by the exigencies of the therapeutic situation. Administering also includes contacting the agent with the LasR protein outside of an organism such as with a culture of bacteria.

The agent can be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the agent can be coated with a material to protect the agent from the action of enzymes, acids and other natural conditions which may inactivate the agent.

The agent can also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable

solutions or dispersions. In all cases, the composition must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the agent in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the agent into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above.

The agent can be orally administered, for example, with an inert diluent or an assimilable edible carrier. The agent and other ingredients can also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the individual's diet. For oral therapeutic administration, the agent can be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations can, of course, be varied and can conveniently be between about 5 to about 80% of the weight of the unit.

The amount of agent in such therapeutically useful compositions is such that a suitable dosage will be obtained.

The tablets, troches, pills, capsules and the like can also contain the following: a binder such as gum gragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it can contain, in addition to materials of the above type, a liquid carrier. Various other materials can be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules can be coated with shellac, sugar or both. A syrup or elixir can contain the agent, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the agent can be incorporated into sustained-release preparations and formulations.

The language "pharmaceutically acceptable carrier" is intended to include solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the agent, use thereof in the therapeutic compositions and methods of treatment is contemplated. Supplementary active compounds can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the individual to be treated; each unit containing a predetermined quantity of agent is calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the agent and the

particular therapeutic effect to be achieve, and (b) the limitations inherent in the art of compounding such an agent for the treatment of *P. aeruginosa* infection in individuals.

The principal agent is compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in an acceptable dosage unit. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

The language "an immunocompromised host" is intended to include an organism that has an immune system that is incapable of reacting to pathogens. The host can be immunocompromised due to a genetic disorder, disease or drugs that inhibit immune response. An immunocompromised host includes an individual afflicted with cystic fibrosis or who is taking corticosteroids or immunosuppressive agents.

The language "infected with *Pseudomonas aeruginosa*" is intended to include an organism that is found to have the bacteria, *Pseudomonas aeruginosa*, present in its body in an infective form. For example, *Pseudomonas aeruginosa* often infects the lungs of cystic fibrosis patients. Even a small number of *Pseudomonas aeruginosa* found in an organism can constitute infection with *Pseudomonas aeruginosa*.

The present invention further pertains to a culture medium containing as an added compound N-(3-oxododecanoyl)homoserine lactone at a concentration effective to stimulate or promote cellular metabolism, growth or recovery. For example, the culture medium could be used to support growth of *Pseudomonas aeruginosa*.

The language "culture medium" is intended to include a substance on which or in which cells grow. The autoinducer molecule can be included in commercially available cell culture media. Culture media include broths, agar, and gelatin.

The present invention also pertains to a method of regulating the expression of a gene. The method comprises inserting a gene into bacteria chosen for enhancement of gene expression by an agent capable of stimulating the activity of the LasR protein and incubating the bacteria with

an agent capable of stimulating the activity of the LasR protein. The method further can include the steps of allowing the gene expression to reach a desired level and then incubating the bacteria with an agent capable of inhibiting the activity of the LasR protein.

The present invention also pertains to a method for detecting the presence or absence of *Pseudomonas aeruginosa* in a sample. The method includes the steps of obtaining a sample fluid suspected of containing *Pseudomonas aeruginosa* and detecting the presence or absence of the autoinducer molecule as an indication of the presence or absence of *Pseudomonas aeruginosa* in the sample. The presence or absence of the autoinducer molecule can be detected using the bioassay described below.

The present invention also pertains to analogs of the autoinducer molecule that inhibit the induction of virulence factors by the autoinducer molecule or LasR. The virulence factors include exotoxin A, elastolytic proteases, and an alkaline protease.

The invention is further illustrated by the following non-limiting examples. The contents of all of the references, published patent applications, and issued patents cited throughout this application are expressly incorporated by reference.

EXAMPLES

Materials And Methods

Bacterial Strains, Plasmids and Culture Conditions

The *E. coli* strains used were TBI (Gibco-Bethesda Research Laboratories Life Technologies (1984) *Focus* 6, 4), MG4 (Railing, G., Bodrug, S. and Linn, T. (1985) *Mol. Gen. Genet.* 201, 379-386), and VJS533 (Stewart, V. J. and Paroles, J.V., Jr. (1988) *J. Bacteriol.* 170, 1589-1597). The *P. aeruginosa* strains used were PAO1, which contains functional *lasR* and *lasI* genes, and PAO-RI, which is a *lasR-lasI*-mutant derived from PAO1 (Gambello, M.J. and Iglewski, B.H. (1991) *J. Bacteriol.* 173, 3000-3009). The plasmids used were pLasI-1, a *lasI* expression vector (Passador, L., Cook, J.M., Gambello, M.J., Rust, L. and Iglewski, B.H. (1993) *Science* 260, 1127-1130), pKDT17, which contains a *lasB::lacZ* reporter of *lasB* promoter activity and *lasR* under control of the *lac* promoter,

and pHV200I⁻, which contains the *V. fischeri* luminescence gene cluster with an inactive *luxI*. These plasmids are all ColEl replicons containing an ampicillin-resistance marker. Construction of pKDT17 involved cloning into SmaI-digested pUCP18 (Schweizer, H.P. (1991) *Gene* 97, 109-112) an 800-bp *lasR* fragment from pMJG1.7 (Gambello, M.J. and Iglewski, B.H. (1991) *J. Bacteriol.* 173, 3000-3009), which extended from the EcoRV site 59-bp upstream of the *lasR* transcriptional start to the AluI site 22bp beyond the *lasR* translational stop codon to construct pKDT11. In this plasmid, *lasR* is under control of the *lac* promoter. An intermediate construct containing only two PvuII sites was made by subcloning the 800-bp fragment in pUC18 to form pKDT13. This intermediate construct was digested with PvuII and the p*lac-lasR* fragment was cloned in TthIII-digested pTS400 (Brumlik, M.J. and Storey, D.G. (1992) *Molec. Microbiol.* 6, 337-344). The resulting plasmid was called pKDT17. The plasmid, pHV200I⁻ was derived from the *lux* regulon-containing pHV200 (Gray, K.M. and Greenberg, E.P. (1992) *J. Bacteriol.* 174, 4384-4390) by introducing a frameshift mutation in *luxI*. This was accomplished by digestion of pHV200 with BglII, filling in the single-stranded overhangs with taq polymerase and treating with T4 DNA ligase.

For production of PAI, cultures of *P. aeruginosa* PAO1 or *E. coli* TB1 containing pLasI-l were grown to the late-logarithmic phase in A medium (Maniatis, T., Fritsch, E.F., and Sambrook, J. (1992) *Molecular Cloning: A Laboratory Manual,* ed. Nolan C. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), 2nd Ed) supplemented with 0.4% glucose, 0.05% yeast extract, and 1 mM $MgSO_4$ with shaking at 37°C, unless otherwise specified. For subsequent use in autoinducer bioassays, *E. coli* MG4 containing pKDT17 was grown in supplemented A medium at 30°C with shaking, and *E. coli* VJS533 containing pHV200I⁻ was grown in L broth (Silhavy, T.J., Berman, M.L. and Enquist, L.W. (1984) *Experiments with Gene Fusions* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), P. 217) at 30°C with shaking. For plasmid screening and maintenance, ampicillin (100 μg/ml) was included in *E. coli* cultures, and carbenicillin (200 ug/ml) was included in cultures of *P. aeruginosa.*

Plasmid Purification and Transformation Procedure

Plasmids were purified and manipulated (Maniatis, T., Fritsch, E.F., and Sambrook, J. (1992) *Molecular Cloning: A Laboratory Manual,* ed. Nolan C. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), 2nd Ed) as described elsewhere. The transformation procedure used was described by Hanahan (Hanahan, D. (1983) *J. Mol. Biol.* 166, 557-580).

Autoinducer Bioassays

The quantitative assay that was developed for PAI was based on a previous report that *E. coli* MG4 containing a plasmid with *lasR* and a *lasB::lacZ* transcriptional fusion showed a 25-fold induction of β -galactosidase, when grown in a medium in which a recombinant *E. coli* containing *lasI* had been grown previously as compared to a medium in which *E. coli* without *lasI* had been grown. It has been found that this PAI activity could be extracted and concentrated in ethyl acetate as described for N-(3-oxohexanoyl)homoserine lactone, the *V. fischeri* autoinducer or VAI (Eberhard, A., Burlingame, A.L., Eberhard, C., Kenyon, G.L., Nealson, K.H.and Oppenheimer, N.J. (1981) *Biochemistry* 20, 2444-2449; Nealson, K.H. (1977) *Arch. Microbiol.* 112, 73-79). For the bioassay, overnight cultures of *E. coli* containing the *lasB*-promoter reporter, pKDT17 were diluted in supplemented A medium to an optical density of 0.1 at 660 nm, and stored on ice. Each bioassay consisted of 2 ml of the cell suspension plus the test sample. After 5.5 h at 30°C with shaking, β-galactosidase activity was measured by the $CHCl_3$-sodium dodecyl sulfate method described by Miller (Miller, J.A. (1976) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), pp. 352-355). As described in Example 1, there was a linear dose response to autoinducer in this bioassay. Without addition of autoinducer, β-galactosidase activities were 25±10 Miller units, and with saturating amounts of autoinducer, β-galactosidase activities were 1300±200 Miller units. A unit of *P. aeruginosa* autoinducer activity is defined as that amount required to achieve 1/2-saturation in the bioassay.

The quantitative assay for VAI was based on those described elsewhere (Kaplan, H.B. and Greenberg, E.P. (1985) *J. Bacteriol.* 163, 1210-1214; Bainton, N.J., Bycroft, B.W., Chhabra, S.R., Stead, P., Gledhill,

L., Hill, P.J., Rees, C. E. D., Winson, M.K., Salmond, G. P. C., Stewart, G. S. A. B. and Williams, P. (1992) *Gene* 116, 87-91; Nealson, K H. (1977) *Arch. Microbiol.* 112, 73-79). *E. coli* VJS533 containing pHV200I$^-$ was used to test for *V.fischeri* autoinducer activity. The plasmid, pHV200I$^-$ contains all of the *V. fischeri* genes necessary for autoinducible luminescence in *E. coli,* however, the gene encoding autoinducer synthase, *luxI,* is inactivated such that *E. coli* containing pHV200I$^-$ is not luminescent without addition of VAI. Overnight cultures of *E. coli* containing pHV200I$^-$ were diluted to an optical density of 0.01 at 660 nm in an assay medium consisting of 0.05% tryptone, 0.03% glycerol, 100 mM NaCl, 50 mM $MgSO_4$, and 10 mM potassium phosphate, pH 7. Each bioassay consisted of 1 ml of the cell suspension plus the test sample. After 3 hours at room temperature, luminescence was measured by using a Beckman LS 1800 Scintillation Counter that was set for single photon counting. Synthetic VAI (Kaplan, N.B., Eberhard, A., Widrig, C. and Greenberg, E.P. (1985) *J. Radiolabelled Cmpds. and Pharmaceut.* 22, 387-395) was used to construct a standard curve. A unit of activity is defined as that amount required to achieve a half-maximal response [equivalent to approximately 25 nM N-(3-oxohexanoyl)homoserine lactone].

Purification of the *P. aeruginosa* Autoinducer Produced by *E. coli* Containing pLasI-1

The procedure for PAI purification was based on that described previously for purification of VAI (Eberhard, A., Burlingame, A.L., Eberhard, C., Kenyon, G.L., Nealson, K.H. and Oppenheimer, N.J. (1981) *Biochemistry* 20, 2444-2449). Cells and culture fluid were separated by centrifugation (10,000 x g for 10 min. at 4°C). The culture fluid was then passed through a 0.2 μm pore-size filter, and the filtered material was extracted twice with equal volumes of ethyl acetate plus 0.1 ml/liter glacial acetic acid. The combined extracts were pooled, water was removed with magnesium sulfate, and the ethyl acetate was removed by rotary evaporation at 40-45°C. The residue was dissolved in 6 ml of ethyl acetate. The ethyl acetate was removed by rotary evaporation and the residue was then extracted with 5 ml of ethanol. The ethanol solution was was dried by rotary evaporation and the residue was dissolved in ethyl acetate. The ethyl acetate

was removed and the residue was extracted in 5 ml of ethanol. This ethanol extract was dried, and dissolved in ethyl acetate. Finally, the sample was dried and dissolved in 0.2 ml of methanol. This sample was further purified by High Performance Liquid Chromatography (HPLC) with a C18 reverse phase column (0.46 x 25 cm). The *P. aeruginosa* autoinducer activity was first eluted as a sharp peak at 73 to 78% methanol in a linear 20 to 100% gradient of methanol and water. Fractions constituting this peak were pooled, dried by rotary evaporation and the residue dissolved in ethyl acetate plus acetic acid. The ethyl acetate was removed, the residue was dissolved in 0.1 ml of methanol and this solution was subjected to further purification by HPLC, eluting isocratically with 65% methanol in water. Fractions containing autoinducer activity were dried, dissolved in ethyl acetate plus acetic acid and stored at -20°C.

Chemical Synthesis of *P. aeruginosa* Autoinducer

Synthesis of PAI, N-(3oxododecanoyl)-L-homoserine lactone was similar to that described by Eberhard et al (Eberhard, A., Burlingame, A.L., Eberhard, C., Kenyon, G.L., Nealson, K.H. and Oppenheimer, N.J. (1981) *Biochemistry* 20, 2444-2449) for synthesis of the *V. fischeri* autoinducer, N-(3-oxohexanoyl)homoserine lactone. The major difference was that ethyl 3-oxododecanoate was used instead of ethyl 3-oxohexanoate. The ethyl 3-oxododecanoate was prepared from decanoyl chloride and the dilithio dianion of monoethyl hydrogen malonate as described previously (Wierenga, W. and Skulnick, H.K. (1979) *J. Org. Chem.* 44, 310-31 1). The ethylene glycol ketal of ethyl 3-oxododecanoate was prepared as described for ethyl 3-oxohexanoate (Eberhard, A., Burlingame, A.L., Eberhard, C., Kenyon, G.L., Nealson, K.H. and Oppenheimer, N.J. (1981) *Biochemistry* 20, 2444-2449) except that Dowex-50 sulfonic acid cation exchange resin was used in place of *p*-toluene sulfonic acid as described by Goswami et al (Goswami, A., Beale, J.M., Jr., Chapman, R.L., Miller, D.W. and Rosazza, J.P. (1987) *J. Natural Prod.* 50, 49-54). The sodium salt was prepared as described (Eberhard, A., Burlingame, A.L., Eberhard, C., Kenyon, G.L., Nealson, K.H. and Oppenheimer, N.J. (1981) *Biochemistry* 20, 2444-2449). The sodium 3-oxododecanoate was incubated with equimolar amounts of

L-homoserine lactone HCl (Sigma Chemical Co., St. Louis, Missouri) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (Aldrich, Milwaukee, Wisconsin), and the resulting ethylene glycol ketal of N-(3-oxododecanoyl)-L-homoserine lactone was deprotected by acid treatment (Eberhard, A., Burlingame, A.L., Eberhard, C., Kenyon, G.L., Nealson, K.H. and Oppenheimer, N.J. (1981) *Biochemistry* 20, 2444-2449) to yield N-(3-oxododecanoyl)-L-homoserine lactone. This compound was purified by preparative HPLC using a 20 to 100% methanol gradient as described above. A sharp peak of autoinducer activity eluted at 73 and 78% methanol, exactly where the major peak of natural autoinducer was found to elute. The fractions containing activity were taken to dryness by rotary evaporation, dissolved in ethyl acetate, and this solution was stored at -20°C prior to further analysis.

Spectra

Proton NMR was performed at the University of Iowa College of Medicine NMR Facility on a Varian Unity 500 MHz instrument. Infrared spectroscopy was performed on a Nicolet 205 FTIR. Chemical ionization mass spectrometry was performed at the University of Iowa College of Medicine Mass Spectrometry Facility on a Nermag RIO-10C instrument with a desorption chemical ionization probe. The reagent gas was ammonia. High-resolution fast atom bombardment was performed at the University of Nebraska - Midwest Center for Mass Spectrometry.

Example 1: Extraction and Purification of PAI from Culture Media

Ethyl acetate extracts of *E. coli* (pLasI-l) culture medium or *P. aeruginosa* PAO1 culture medium contained PAI activity in an amount equivalent to that in the culture medium prior to extraction. Bioassays on extracts of media from cultures grown to an equivalent optical density of 0.3 in supplemented A medium indicated the *P. aeruginosa* culture and the *E. coli* (pLasI-l) culture produced roughly equivalent amounts of PAI (Fig. 1).

Because it has been reported that *P. aeruginosa* produces the *V. fischeri* autoinducer, N-(3-oxohexanoyl)homoserine lactone (Bainton, N.J., Bycroft, B.W., Chhabra, S.R., Stead, P., Gledhill, L., Hill, P. J., Rees, C. E.

D., Winson, M.K., Salmond, G. P. C., Stewart, G. S. A. B. and Williams, P. (1992) *Gene* 116, 87-91), this compound was tested for activity as an inducer for the *lasB* promoter. Also, a homolog of the VAI was tested, N-(3-oxooctanoyl)homoserine lactone, which has recently been reported to serve as the autoinducer in conjugal transfer gene activation in the Gram-negative bacterium, *Agrobacterium tumefaciens,* a plant pathogen (Zhang, L., Murphy, P. J., Kerr, A. and Tate, M. (1993) *Nature* 362, 446-448). VAI had no detectable activity when tested in the *P. aeruginosa* autoinducer bioassay at concentrations as high as 1 uM. This compound gives a maximal response at about 50 nM in the *V. fischeri* autoinducer bioassay. The *A. tumefaciens* autoinducer (AAI), however, did show considerable activity (Table 1). This suggested that PAI was N-(3-oxooctanoyl)homoserine lactone or a compound related to it.

TABLE 1. Influence of *P. aeruginosa, V.fischeri, and A. tumeficians* Autoinducers on *lasB* Promoter Activity in *E. coli* MG4 (pKDT17)

Autoinducer added	β-galactosidase (Miller units)[1]
None	26 +/- 2
50 nM N-(3-oxohexanoyl)homoserine lactone (VAI)	33 +/- 3
500 nM N-(3-oxohexanoyl)homoserine lactone (VAI)	34 +/- 1
50 nM N-(3-oxooctanoyl)homoserine lactone (AAI)	56 +/- 5
500 nM N-(3-oxooctanoyl)homoserine lactone (AAI)	733 +/- 50
1.0 unit *P. aeruginosa* autoinducer[2]	735 +/- 110
3.0 units *P. aeruginosa* autoinducer[2]	1470 +/- 90

[1] *P. aeruginosa* autoinducer assays were performed as described in Materials and Methods. β-galactosidase activity is a measure of *lasB::lacZ* promoter activity. Numbers are the average of four experiments ± the ranges.
[2]The *P. aeruginosa* autoinducer was an ethyl acetate extract of the culture medium in which *E. coli* (pLasI-1) was grown.

When PAI from *E. coli* containing pLasI-1 or *P. aeruginosa* PAO1 was subjected to HPLC, a single major peak of activity was observed (Fig. 2). In the case of PAI produced by *E. coli* (pLasI-1) there was a small peak of activity that eluted just after the major peak, and with *P. aeruginosa* there was a small peak of activity that eluted just prior to the major peak. N-(3-oxooctanoyl)homoserine eluted well ahead of the major peak of PAI (Fig. 2). This showed that PAI is not N-(3-oxooctanoyl)homoserine lactone. The *V. fischeri* autoinducer, N-(3-oxohexanoyl)homoserine lactone elutes ahead of N-(3-oxooctanoyl) homoserine lactone. The chromatographic behavior of the PAI, together with the finding that AAI shows activity in the bioassay for *P. aeruginosa* autoinducer suggests that PAI is an

N-acyl-homoserine lactone with a hydrophobic side-chain, which is longer than that of AAI.

Because it has been reported that *P. aeruginosa* produces the *V. fischeri* autoinducer (Bainton, N.J., Bycroft, B.W., Chhabra, S.R., Stead, P., Gledhill, L., Hill, P. J., Rees, C. E. D., Winson, M.K., Salmond, G. P. C., Stewart, G. S. A. B. and Williams, P. (1992) *Gene* 116, 87-91), fractions were tested from HPLC for induction of luminescence by using the *V. fischeri* autoinducer bioassay. In fact, there was a peak of activity that eluted at the same location as synthetic N-(3-oxohexanoyl)homoserine lactone. There was also a peak that eluted at the same location as synthetic N-(3-oxooctanoyl)homoserine lactone, which shows some activity with *V. fischeri* (Eberhard, A., Widrig, C., MacBath, P. and Schineller (1986) *Arch. Microbiol.* 146, 35-40), and, as has been shown, is an inducer of the *P. aeruginosa lasB* (Table 1). However, only low levels of these compounds were present in extracts of either *P. aeruginosa* or *E. coli* (pLasI-l) culture media. There was no significant VAI activity in the PAI peaks shown in Fig. 2. Apparently, this *P. aeruginosa* autoinducer cannot cross-react with the *V. fischeri* LuxR protein to activate the luminescence genes.

Example 2: Analysis of Purified *P. aeruginosa* Autoinducer

The level of PAI activity in extracts of *P. aeruginosa* PAO1 medium was similar to the level in extracts of *E. coli* containing pLasI-l (Fig. 1). The autoinducer produced by *E. coli* containing pLasI-l was purified because *P. aeruginosa* produces a great variety of extracellular compounds (Nicas, T.I. and Iglewski, B.H. (1985) *Can. J. Microbiol.* 31, 387-392) that could complicate purification of the autoinducer. Using the purification procedure described in the Materials and Methods, approximately 300 μg of PAI was obtained from 3 L of culture fluid.

Analysis of the purified PAI by proton NMR in D_2O at 25°C showed a spectrum that was remarkably similar to that for VAI (Eberhard, A., Burlingame, A.L., Eberhard, C., Kenyon, G.L., Nealson, K.H. and Oppenheimer, N.J. (1981) *Biochemistry* 20, 2444-2449) except that the integrations of the methyl triplet at 0.85 ppm, and the CH_2 multiplet at 1.28 ppm indicated the purified PAI had a longer alkyl chain than does VAI.

Based on the similarity between the proton NMR spectrum of PAI and VAI, and the ratio of the methyl triplet and the methylene multiplet, it seemed likely that the purified compound was N-(3-oxododecanoyl)homoserine lactone. Chemical ionization mass spectrometry showed a strong quasimolecular $(M+H)^+$ ion with an *m/z* of 298. This is consistent with the conclusion from the proton NMR analysis that the compound was N-(3-oxododecanoyl)homoserine lactone. The chemical composition was confirmed by high-resolution fast atom bombardment, which more precisely established the mass of the purified compound. The *m/z* of the $(M+H)^+$ was 298.2018. This corresponded to a chemical composition of $C_{16}H_{27}NO_4$. This is the composition of N-(3-oxododecanoyl)homoserine lactone.

Example 3: Analysis of synthetic N-(3-oxododecanoyl)homoserine lactone

As a confirmation of the conclusion that the purified PAI was N-(3-oxododecanoyl)homoserine lactone, this compound was synthesized. The synthetic compound had chromatographic and spectral properties indistinguishable from those of the material purified from culture medium and was biologically active.

The dose response in a *P. aeruginosa* autoinducer bioassay indicates the half-maximal response occurs at 3 to 5 nM N-(3-oxododecanoyl) L-homoserine lactone (Fig. 3). This is in the range found for the *V. fischeri* (Kaplan, H.B. and Greenberg, E.P. (1985) *J. Bacteriol.* 163, 1210-1214) and *A. tumefaciens* (Goswami, A., Beale, J.M., Jr., Chapman, R. L., Miller, D.W. and Rosazza, J.P. (1987) *J. Natural Prod.* 50, 49-54) autoinducer systems where half saturations occur at about 50 nM and 5 nM, respectively.

Discussion

Based on the evidence presented in the above examples, the autoinducer, which serves in conjunction with the LasR protein to activate a number of *P. aeruginosa* virulence genes, is N-(3-oxododecanoyl)homoserine lactone. This autoinducer has a longer acyl side chain than related autoinducers from other bacteria (Fig. 4). It was reported elsewhere that *P. aeruginosa* produces the *V. fischeri* autoinducer,

N-(3-oxohexanoyl)homoserine lactone (Bainton, N.J., Bycroft, B.W., Chhabra, S.R., Stead, P., Gledhill, L., Hill, P.J., Rees, C.E. D., Winson, M.K., Salmond, G. P. C., Stewart, G. S. A. B. and Williams, P. (1992) *Gene* 116, 87-91), and it was suggested that VAI was the inducer required together with LasR for activation of specific *P. aeruginosa* virulence genes (Jones, S., Yu, B., Bainton, N.J., Birdsall, M., Bycroft, B.W., Chhabra, S.R., Cox, A.J.R., Golby, P., Reeves, P.J., Stephens, S., Winson, M.K., Salmond, G.P.C., Stewart G.S.A.B. and Williams, P. (1993) *EMBO J.* 12, 2477-2482). The analysis performed in the above Examples confirmed that a compound, which serves to induce the *V. fischeri lux* genes is produced by the *lasI* gene product, and this compound has the behavior of *V. fischeri* autoinducer in HPLC. However, relatively low levels of this compound were synthesized by *lasI*-containing *P. aeruginosa* or *E. coli* (the concentration of this compound was 0.5% of the PAI concentration in extracts of *P. aeruginosa* PAO1), and neither this compound nor authentic VAI (at concentrations as high as 500 nM) showed *P. aeruginosa* autoinducer activity. In fact, the *lasI* gene product appears to catalyze the synthesis of a number of related compounds by *E. coli* or *P. aeruginosa*, including compounds that behaved as did N-(3-oxohexanoyl)homoserine lactone and N-(3oxooctanoyl)homoserine lactone when subjected to methanol-water gradient HPLC. However, N-(3-oxododecanoyl)homoserine lactone is the most abundantly produced of these related compounds. Based on the data in Figs. 1 and 3, approximately 120 ng/ml of this autoinducer was present in a culture of *P. aeruginosa* grown in supplemented A medium as described. This is equivalent to 400 nM PAI, and is in about 40-fold excess of the concentrations required with LasR to fully activate the *lasB* promoter. In contrast , it is estimated that approximately 0.5 ng/ml of VAI was present in the culture of *P. aeruginosa.*

AAI shows activity not only as the *A. tumefaciens* (Zhang, L., Murphy, P.J., Kerr, A. and Tate, M. (1993) *Nature* 362, 446-448) autoinducer, but also shows activity together with the LuxR protein in activation of *V. fischeri* luminescence genes (Eberhard, A., Widrig, C., MacBath, P. and Schineller (1986) *Arch. Microbiol.* 146, 35-40), and together with the LasR protein in activation of the *P. aeruginosa lasB* (Table 1). The concentration of AAI required for activity as the *P. aeruginosa* autoinducer

was higher than the concentration of PAI required for activity, and the maximal response was lower (Table 1, Fig. 3). VAI, N-(3-oxohexanoyl)homoserine lactone, did not show any detectable activity as a *P. aeruginosa* autoinducer (Table 1). Apparently, the LasR protein shows specificity with respect to the N-acyl side chain length. This also has been shown for the *V. fischeri* LuxR protein (Eberhard, A., Widrig, C., MacBath, P. and Schineller (1986) *Arch. Microbiol.* 146, 35-40) and the *A. tumefaciens* TraR (Zhang, L., Murphy, P.J., Kerr, A. and Tate, M. (1993) *Nature* 362, 446-448).

It has recently become apparent that regulatory circuits homologous to the LuxR-LuxI regulatory circuit in *V. fischeri* and the LasR-LasI circuit in *P. aeruginosa* are common to a number of diverse Gram-negative bacteria. At this time, four different autoinducer structures (including PAI as described here) are known (Fig. 4). The luminescence genes in *Vibrio harveyi* are controlled by N-(3-hydroxybutanoyl)homoserine lactone or N-(β-hydroxybutyryl)homoserine lactone (Cao, J.-G. and Merghen, E.A. (1989) *J. Biol. Chem.* 264, 21670-21676; Cao, J.-G. and Meighen, E.A. (1993) *J. Bacteriol.* 175, 3856-3862) but homologs of *luxI* and *luxR* in this organism have not been identified. A number of bacteria have been reported to produce N-(3-oxohexanoyl)homoserine lactone, VAI (Bainton, N.J., Bycroft, B.W., Chhabra, S.R., Stead, P., Gledhill, L., Hill, P. J., Rees, C.E.D., Winson, M.K., Salmond, G.P. C., Stewart, G.S.A.B. and Williams, P. (1992) *Gene* 116, 87-91). In one of these bacteria at least, *Erwinia carotovora,* the *luxI* and *luxR* homologs *expI* and *expR* have been identified (Pirhonen, M., Flego, D., Heikinheimo, R. and Palva, E.T. (1993) *EMBO J.* 12, 2467-2476). The *expI* gene directs the synthesis of an autoinducer that is required together with the *expR* product for induction of extracellular protease in a fashion reminiscent of PAI control of extracellular protease induction in *P. aeruginosa.* Conjugal transfer genes in *A. tumifaciens* are controlled by AAI (Zhang, L., Murphy, P.J., Kerr, A. and Tate, M. (1993) *Nature* 362, 446-448) together with a transcriptional activator encoded by *traR* (Piper, K.R., von Bodman, S.B. and Farrand, S.K. (1993) *Nature* 362, 448-450). The gene or genes required for AAI synthesis have not yet been described.

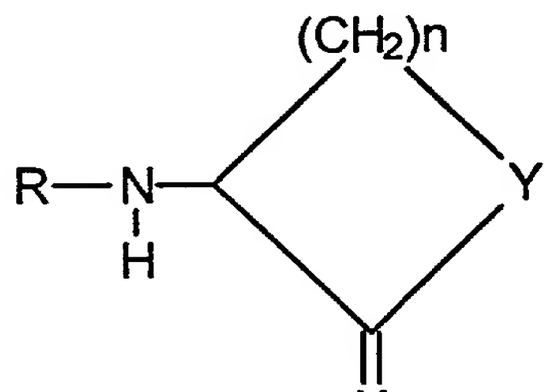
EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific embodiments described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

CLAIMS

1. N-(3-oxododecanoyl)homoserine lactone.

2. An autoinducer molecule comprising a molecule of the formula:



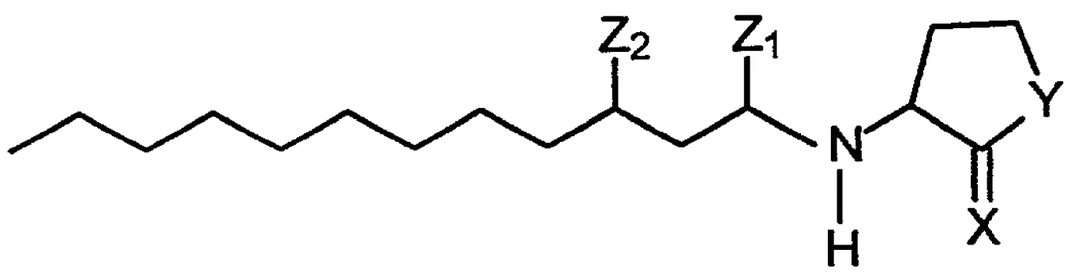
wherein n is 2 or 3; Y is O, S, or NH; X is O, S, or NH; and R is a fatty hydrocarbon or acyl moiety that may be substituted or a moiety having at least seven members containing a ring structure that may be substituted; the molecule being able to regulate the activity of the LasR protein of *Pseudomonas aeruginosa*.

3. The autoinducer molecule of claim 2 wherein R is a C_7 - C_{14} acyl moiety.

4. The autoinducer molecule of claim 3 wherein R is a C_{10} or higher acyl moiety.

5. The autoinducer molecule of claim 4 wherein R is a C_{12} acyl moiety.

6. The autoinducer molecule of claim 5 wherein the molecule is of the formula



wherein X and Y are as defined above and Z_1 and Z_2 are independently selected from the group consisting of hydrogen, **=O**, **=S**, and **=NH**; the molecule being able to regulate gene expression.

7. The autoinducer molecule of claim 6 wherein the molecule is N-(3-oxododecanoyl)homoserine lactone.

8. The autoinducer molecule of claim 2 wherein R contains a heterocyclic ring structure.

9. The autoinducer molecule of claim 8 wherein the heterocyclic ring structure has five to seven ring members.

10. The autoinducer molecule of claim 9 wherein the heterocyclic ring structure contains oxygen.

11. The autoinducer molecule of claim 2 wherein R contains a carbocyclic ring structure.

12. The autoinducer of claim 11 wherein the carbocyclic ring structure is a fused ring system.

13. The autoinducer molecule of claim 2 wherein the molecule is purified from the native source.

14. The autoinducer molecule of claim 13 wherein the native source is the culture media of *Pseudomonas aeruginosa*.

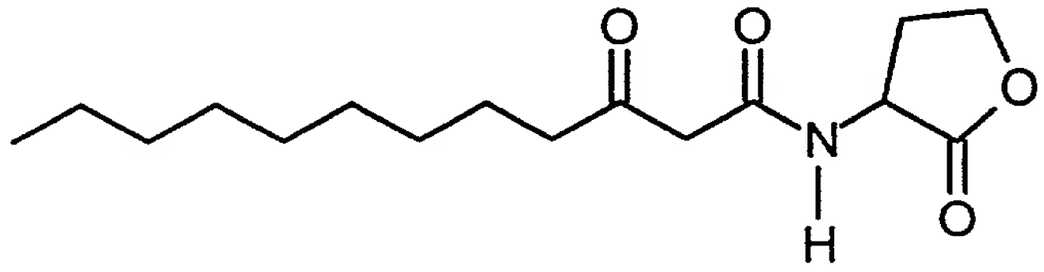
15. The autoinducer molecule of claim 2 wherein the molecule is synthesized by chemical means.

16. The autoinducer molecule of claim 2 wherein the molecule is an optically active isomer.

17. The autoinducer molecule of claim 16 wherein the isomer is the L-isomer.

18. The autoinducer molecule of claim 16 wherein the isomer is the D-isomer.

19. An autoinducer molecule comprising a molecule of the formula:



the molecule being able to regulate gene expression.

20. The autoinducer molecule of claim 19 wherein the gene expression within bacteria is regulated.

21. An analog of N-(3-oxododecanoyl)homoserine lactone that affects the activity of the LasR protein.

22. The analog of claim 21 wherein the analog inhibits the autoinducer activity of the N-(3-oxododecanoyl)homoserine lactone.

23. The analog of claim 21 wherein the analog synergistically enhances the autoinducer activity of N-(3-oxododecanoyl)homoserine lactone.

24. The analog of claim 21, wherein the analog is an agonist of the LasR protein of *Pseudomonas aeruginosa*.

25. The analog of claim 21, wherein the analog is an antagonist of the LasR protein of *Pseudomonas aeruginosa*.

26. A method of selecting inhibitors of the autoinducer molecule of *Pseudomonas aeruginosa* comprising:

contacting the autoinducer molecule with a suspected inhibitor;

measuring the ability of the treated autoinducer molecule to stimulate the activity of a selected gene;

determining whether the suspected inhibitor inhibits the ability of the autoinducer molecule to stimulate the activity of a selected gene; and

selecting the suspected inhibitors that inhibit the autoinducer molecule.

27. A method of selecting synergists of the autoinducer molecule of *Pseudomonas aeruginosa* comprising:

contacting the autoinducer molecule with a suspected synergist;

measuring the ability of the treated autoinducer molecule to stimulate the activity of a selected gene;

determining whether the suspected synergist enhances the ability of the autoinducer molecule to stimulate the activity of a selected gene; and

selecting the suspected synergists that enhance the activity of the autoinducer molecule.

28. A therapeutic composition comprising an agent having the ability to inhibit the activity of the LasR protein of *Pseudomonas aeruginosa* and a pharmaceutically acceptable carrier.

29. The therapeutic composition of claim 28 wherein the agent is a molecule which inhibits the autoinducer activity of N-(3-oxododecanoyl)homoserine lactone.

30. A method of inhibiting the infectivity of *Pseudomonas aeruginosa* comprising administering to an individual a therapeutically effective amount of an agent that inhibits the activity of the LasR protein.

31. A method of treating an immunocompromised individual infected with *Pseudomonas aeruginosa* comprising administering to the individual a therapeutically effective amount of an agent that inhibits the activity of the LasR protein.

32. A method of claim 31 wherein the immunocompromised individual is afflicted with cystic fibrosis.

33. A culture medium containing as an added compound N-(3-oxododecanoyl)homoserine lactone at a concentration effective to stimulate or promote cellular metabolism, growth, or recovery.

34. The culture medium of claim 33 wherein the cellular growth of *Pseudomonas aeruginosa* is stimulated or enhanced.

35. A method of regulating the expression of a gene comprising:

inserting a gene into bacteria chosen for enhancement of gene expression by an agent that enhances the activity of the LasR protein; and

incubating the bacteria with an agent that enhances the activity of the LasR protein such that the expression of the gene is regulated.

36. The method of claim 35 wherein the method further comprises the additional steps of:

allowing the gene expression to reach a desired level; and

incubating the bacteria with an agent that inhibits the activity of the LasR protein regulating the gene expression by the bacteria.

37. A method of regulating the expression of a gene comprising:

inserting a gene into a cell chosen for enhancement of gene expression by N-(3-oxododecanoyl)homoserine lactone; and

incubating the cell with N-(3-oxododecanoyl)homoserine lactone such that the expression of the gene is regulated.

38. The method of claim 37 wherein the method further comprises the additional steps of:

allowing the gene expression to reach a desired level; and

incubating the cell with an agent that inhibits the activity N-(3-oxododecanoyl)homoserine lactone regulating the gene expression by the cell.

39. An inhibitor of the autoinducer activity of N-(3-oxododecanoyl)homoserine lactone.

40. An analog of N-(3-oxododecanoyl)homoserine lactone that inhibits the induction of virulence factors by N-(3-oxododecanoyl)homoserine lactone or LasR.

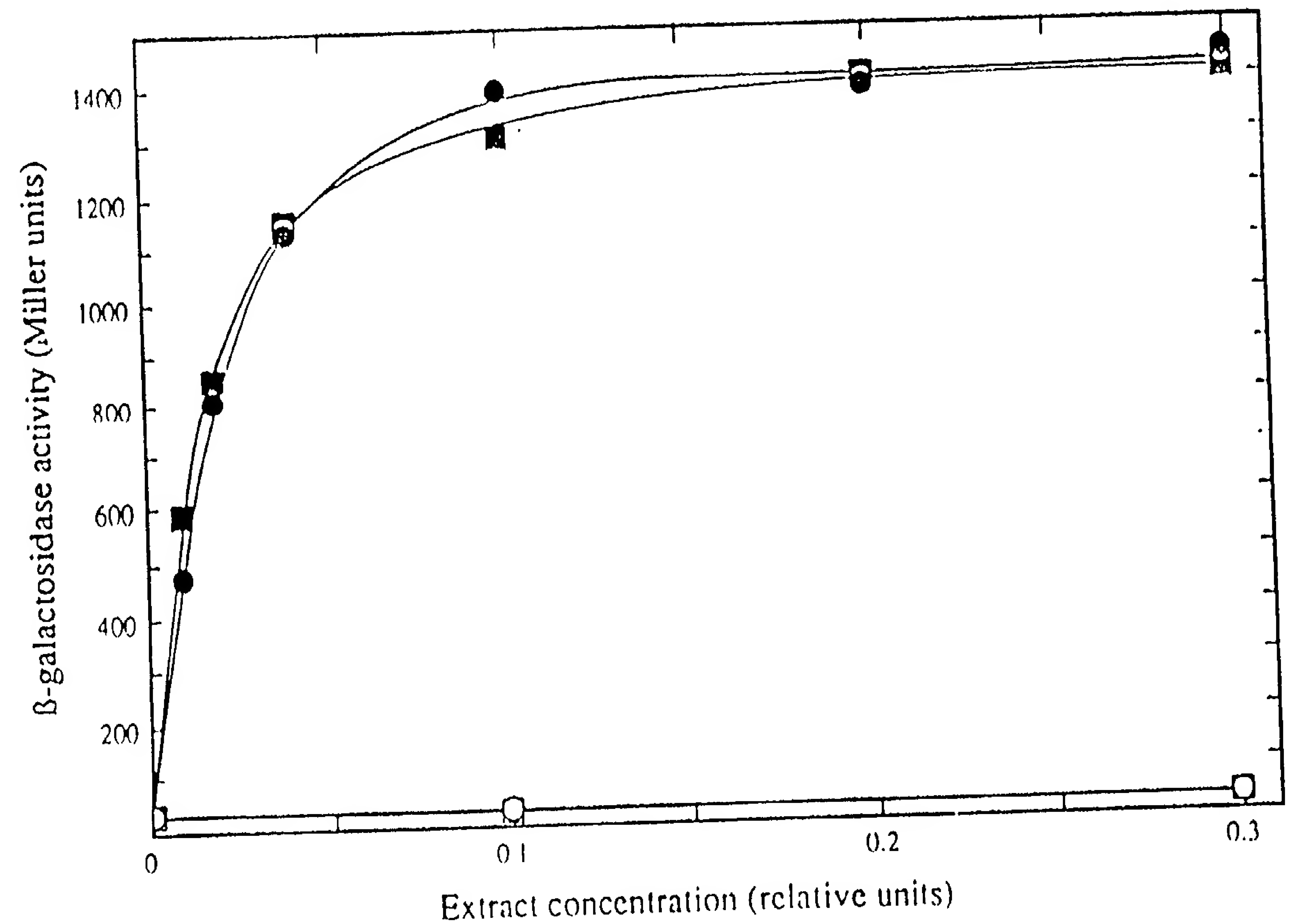
41. The analog of claim 40 wherein the virulence factor is exotoxin A.

42. The analog of claim 40 wherein the virulence factor is an elastolytic protease.

43. The analog of claim 40 wherein the virulence factor is an alkaline protease.

ABSTRACT

Autoinducer molecules, e.g., N-(3-oxododecanoyl)homoserine lactone, for *Pseudomonas aeruginosa* are described. The molecules regulate gene expression in the bacterium. Therapeutic compositions and therapeutic methods involving analogs and/or inhibitors of the autoinducer molecules also are described. The molecules are useful for treating or preventing infection by *Pseudomonas aeruginosa*.



ß-galactosidase activity (Miller units)
1400
1200
1000
800
600
400
200
0
0.1
0.2
0.3
Extract concentration (relative units)

FIGURE 1

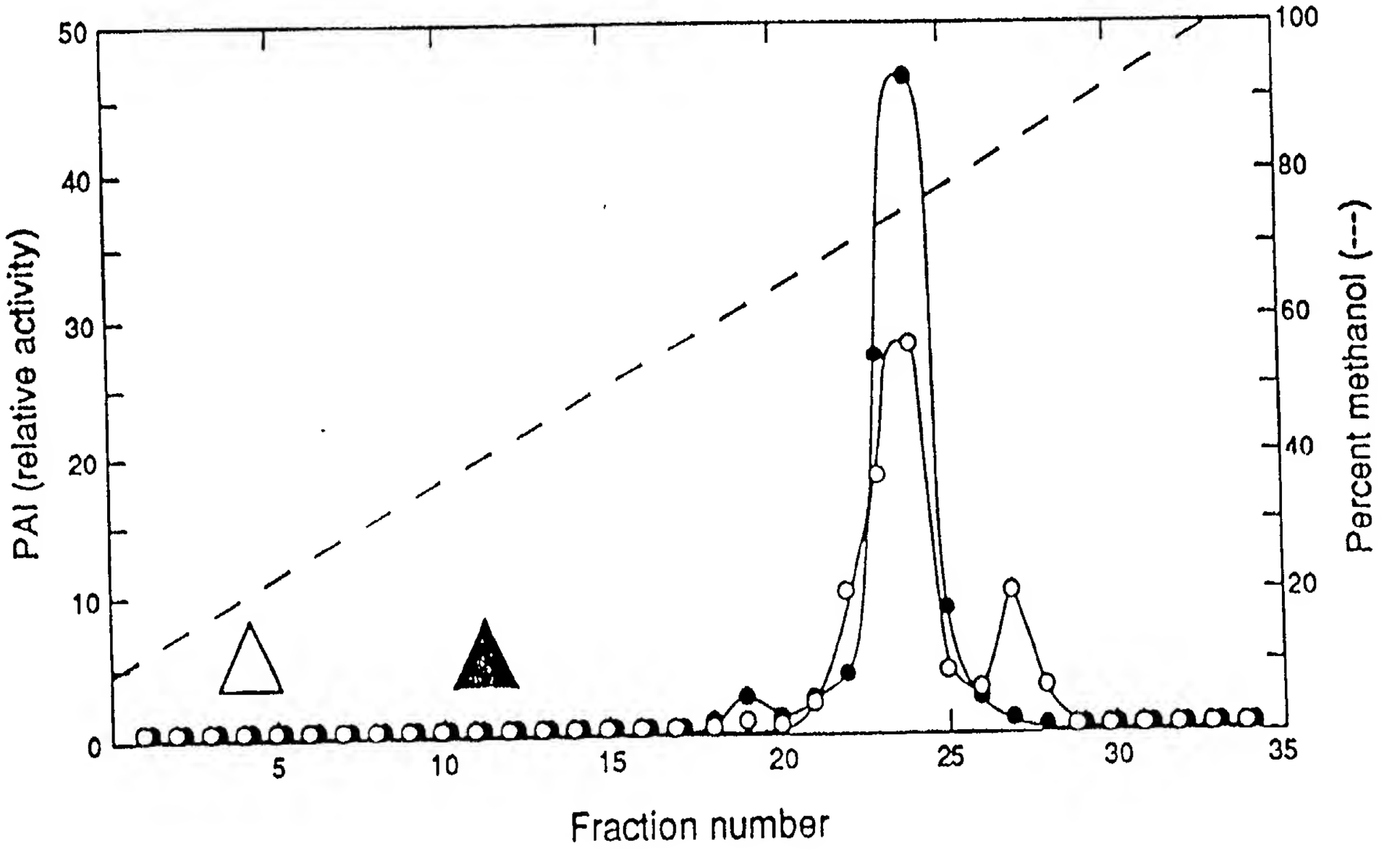


FIGURE 2

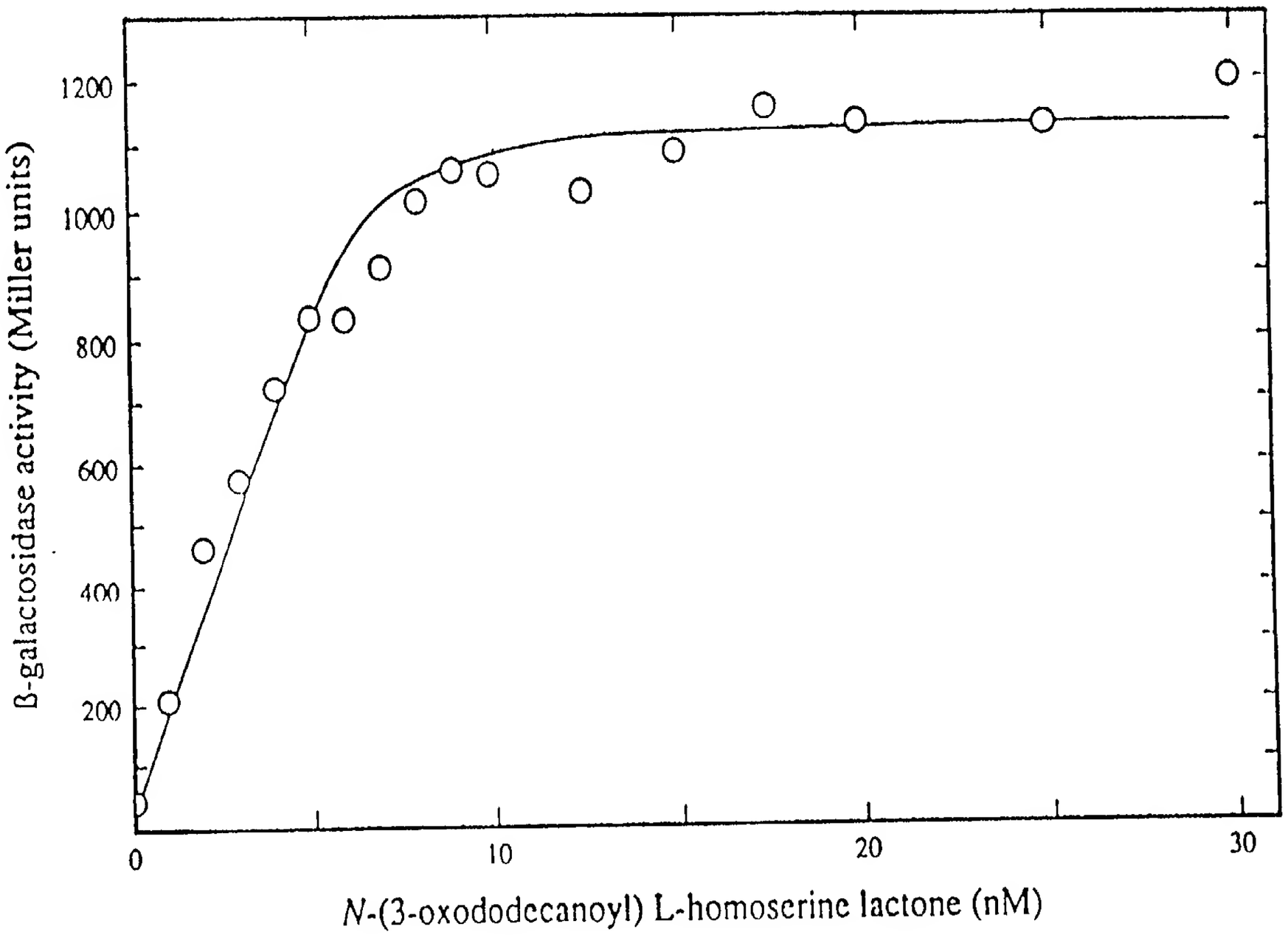
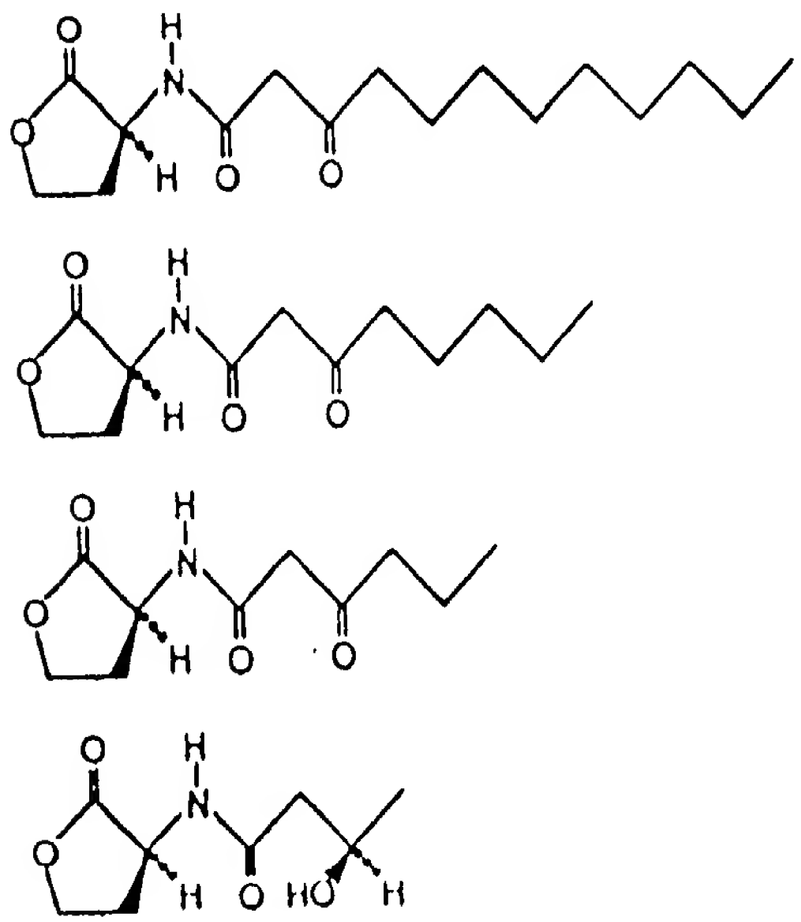

ß-galactosidase activity (Miller units)
1200
1000
800
600
400
200
0
10
20
30
N-(3-oxododecanoyl) L-homoserine lactone (nM)

FIGURE 3

FIGURE 4



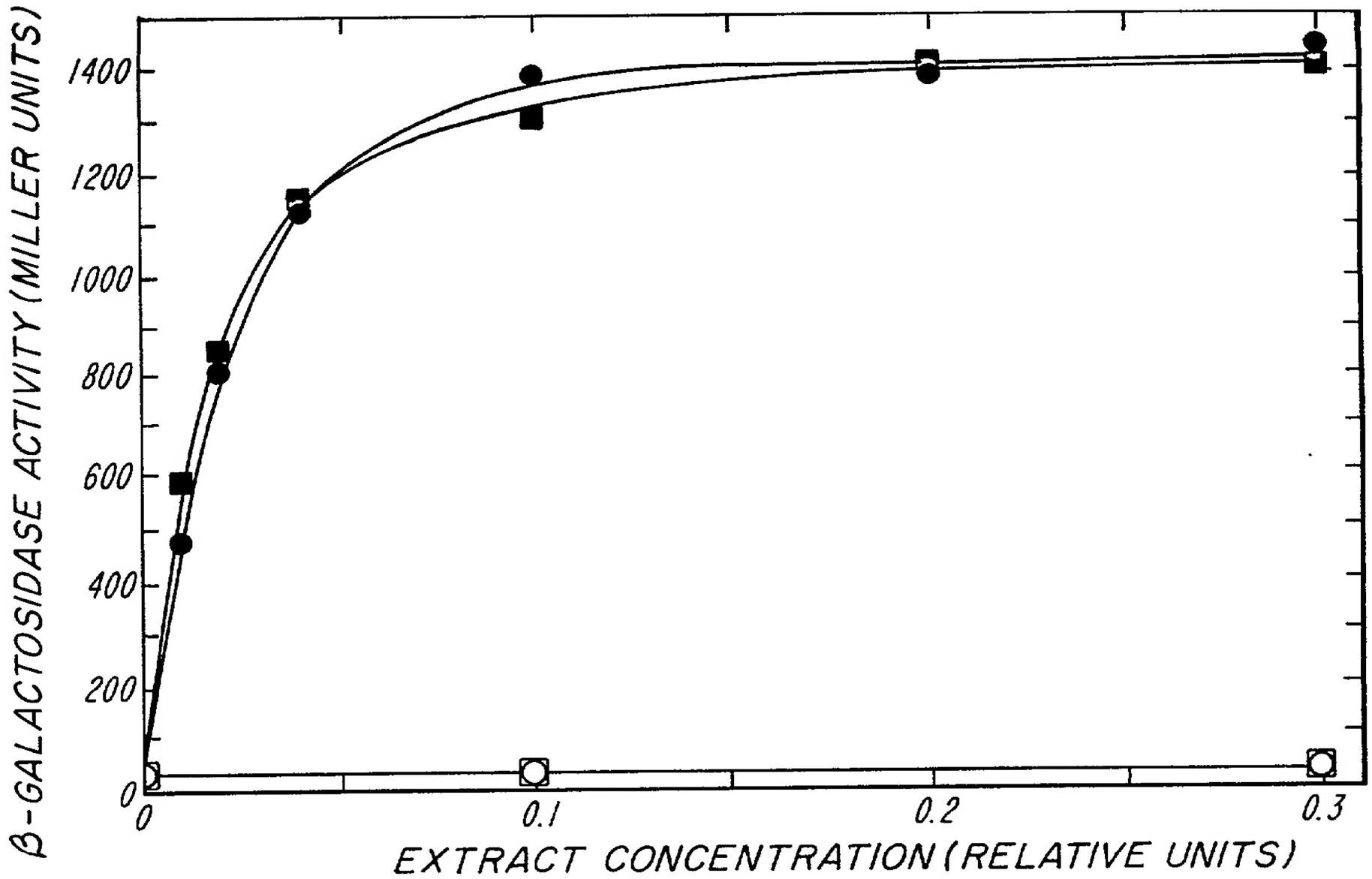
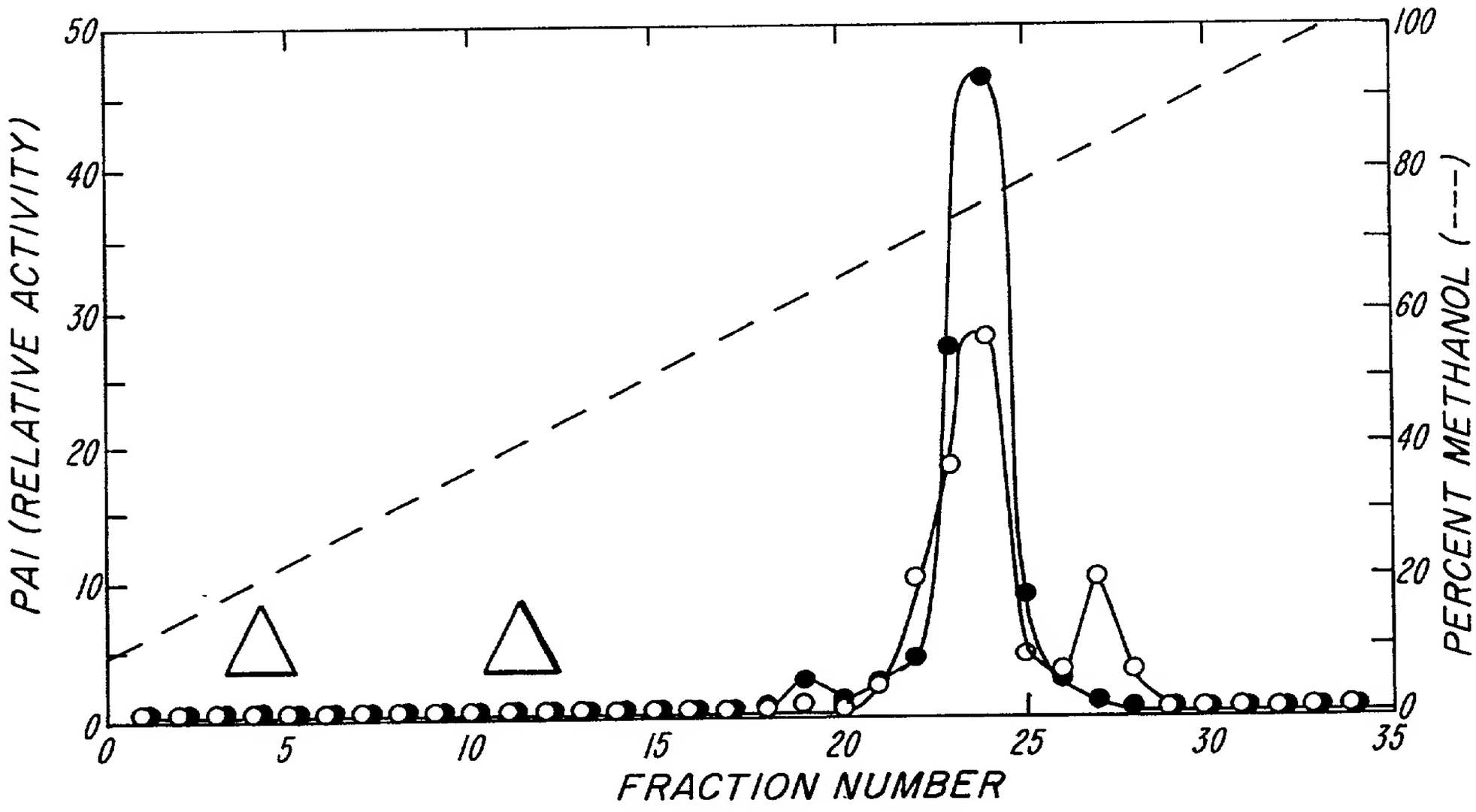

β-GALACTOSIDASE ACTIVITY (MILLER UNITS)
1400
1200
1000
800
600
400
200
0
0
0.1
0.2
0.3
EXTRACT CONCENTRATION (RELATIVE UNITS)

FIG. 1


PAI (RELATIVE ACTIVITY)
50
40
30
20
10
0
0
5
10
15
20
25
30
35
FRACTION NUMBER
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80
60
40
20
0
PERCENT METHANOL (---)

FIG. 2

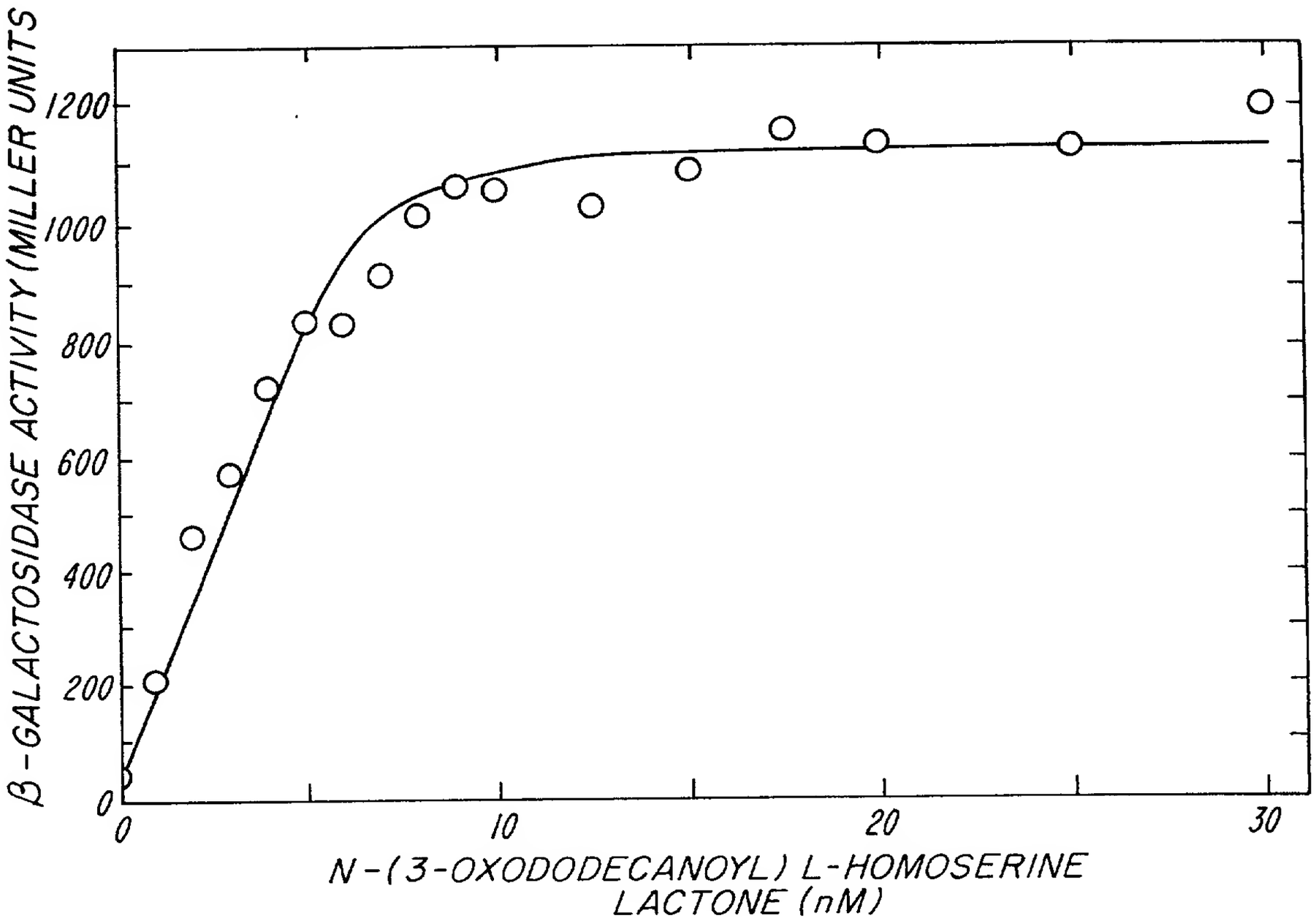
β-GALACTOSIDASE ACTIVITY (MILLER UNITS
1200
1000
800
600
400
200
0
0
10
20
30
N-(3-OXODODECANOYL) L-HOMOSERINE LACTONE (nM)

FIG. 3

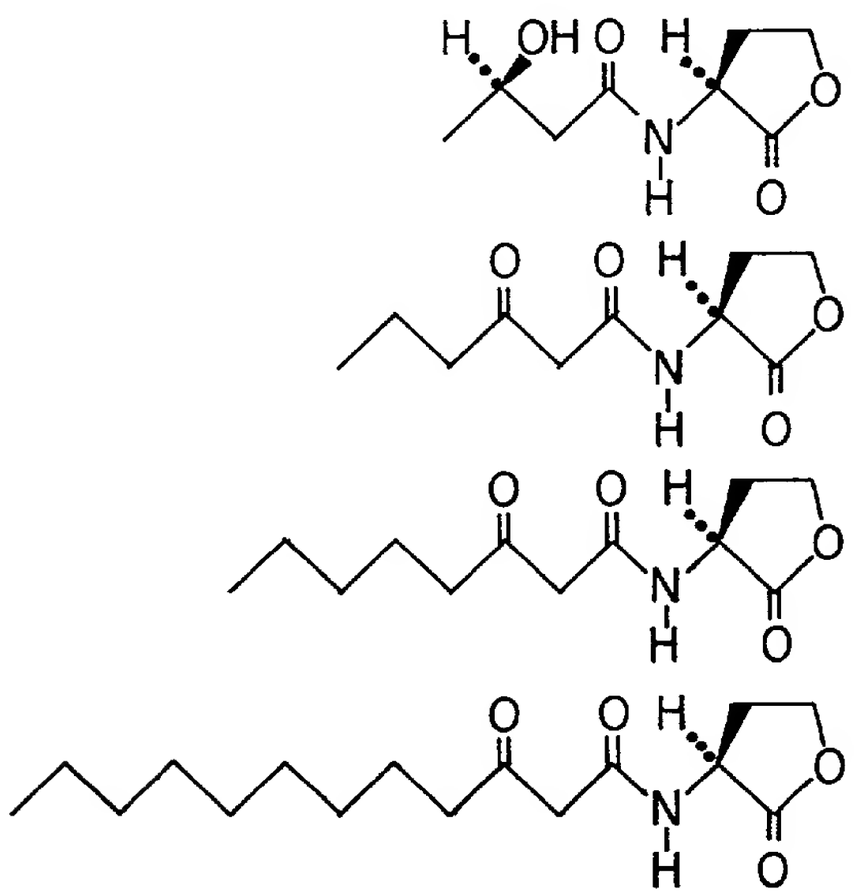
H
OH
O
H
N
H
O
O

FIG. 4

Attorney's
Docket
Number UIZ-003

Declaration, Petition and Power of Attorney For Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

AN AUTOINDUCER MOLECULE

the specification of which

(check one)

_ is attached hereto.

X was filed on August 9, 1993 as

Application Serial No. 104,487

and was amended on _____.
(if applicable)

I do not know and do not believe that the subject matter of this application was ever known or used in the United States before my invention thereof or patented or described in any printed publication in any country before my invention thereof or more than one year prior to the date of this application, and that said subject matter has not been patented or made the subject of an issued inventor's certificate in any country foreign to the United States on an application filed by me or my legal representatives or assigns more than twelve months prior to the date of this application; that I acknowledge my duty to disclose information of which I am aware which is material to the examination of this application, that no application for patent or inventor's certificate on the subject matter of this application has been filed by me or my representatives or assigns in any country foreign to the United States, except those identified below, and that I have reviewed and understand the contents of the specification, including the claims as amended by any amendment referred to herein.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

PRIORITY CLAIM

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

Check one:

X no such applications have been filed.

_ such applications have been filed as follows

EARLIEST FOREIGN APPLICATION(S), IF ANY FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

Country	Application Number	Date of Filing (month,day,year)	Priority Claimed Under 35 USC 119
			_ Yes No _
			_ Yes No _
			_ Yes No _
			_ Yes No _
			_ Yes No _

ALL FOREIGN APPLICATION(S), IF ANY FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

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(Application Serial No.)	(Filing Date)	(Status) (patented,pending,aband.)
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Giulio A. DeConti, Jr.	Reg. No. 31,503		

Send Correspondence to:

Elizabeth A. Hanley, Lahive & Cockfield, 60 State Street, Boston, Massachusetts 02109

Direct Telephone Calls to: (name and telephone number)

Elizabeth A. Hanley, (617) 227-7400

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Full name of sole or first inventor James P. Pearson
Inventor's signature Date
Residence 412 South Linn Street, Apartment 12, Iowa City, Iowa 52240
Citizenship American
Post Office Address (if different)

Full name of second inventor, if any Kendall M. Gray
Inventor's signature Date
Residence 120½ Fairchild, Iowa City, Iowa 52245
Citizenship American
Post Office Address (if different)

Full name of third inventor, if any Luciano Passador
Inventor's signature Date
Residence 604-12 Suburban Court, Rochester, New York 14620
Citizenship Canadian
Post Office Address (if different)

Full name of fourth inventor, if any Kenneth D. Tucker
Inventor's signature Date 10-6-93
Residence 20408 Afternoon Lane, Germantown, Maryland 20874
Citizenship American
Post Office Address (if different)

Full name of fifth inventor, if any Anatol Eberhard
Inventor's signature Date
Residence 2434 Cottington Road, Brooktondale, New York 14817
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Full name of sixth inventor, if any Barbara H. Iglewski	
Inventor's signature	Date
Residence 8 McCoord Woods, Fairport, New York 14450	
Citizenship American	
Post Office Address (if different)	

Full name of seventh inventor, if any Everett P. Greenberg	
Inventor's signature	Date
Residence 4020 Stewart Street, Iowa City, Iowa 52240	
Citizenship American	
Post Office Address (if different)	

Attorney's
Docket
Number UIZ-003

Declaration, Petition and Power of Attorney For Patent Application

As a below named inventor, I hereby declare that:

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I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

AN AUTOINDUCER MOLECULE

the specification of which

(check one)

_ is attached hereto.

X was filed on August 9, 1993 as

Application Serial No. 104,487

and was amended on _____.
(if applicable)

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Inventor's signature Date
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Citizenship American
Post Office Address (if different)

Full name of third inventor, if any Luciano Passador
Inventor's signature Date Luciano Passador Oct 7th, 1993
Residence 604-12 Suburban Court, Rochester, New York 14620
Citizenship Canadian
Post Office Address (if different)

Full name of fourth inventor, if any Kenneth D. Tucker
Inventor's signature Date
Residence 20408 Afternoon Lane, Germantown, Maryland 20874
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Inventor's signature Date
Residence 2434 Cottington Road, Brooktondale, New York 14817
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Post Office Address (if different)

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Inventor's signature Date Oct 7, 1993
Residence 8 McCoord Woods, Fairport, New York 14450
Citizenship American
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Full name of seventh inventor, if any Everett P. Greenberg
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Residence 4020 Stewart Street, Iowa City, Iowa 52240
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Attorney's
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the specification of which

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Inventor's signature James Philip Pearson Date Oct. 6, 1993
Residence 412 South Linn Street, Apartment 12, Iowa City, Iowa 52240
Citizenship American
Post Office Address (if different)

Full name of second inventor, if any Kendall M. Gray
Inventor's signature Date Kendall M. Gray 10/6/93
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Inventor's signature Date
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Full name of seventh inventor, if any Everett P. Greenberg
Inventor's signature Date Everett P. Greenberg Oct. 6, 1995
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Inventor's signature Date 7 Oct 1993
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Inventor's signature Date
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Full name of seventh inventor, if any Everett P. Greenberg
Inventor's signature Date
Residence 4020 Stewart Street, Iowa City, Iowa 52240
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Post Office Address (if different)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: Pearson, James P. *et al.*	Group Art Unit: 1641
Serial No.: Not Yet Assigned	Examiner: Baskar, P.
Filed: Herewith (continuation application of 08/456,864	
For: Autoinducer Molecule	
Attorney Docket No.: UIZ-003DVCN	

Assistant Commissioner for Patents
Washington, D.C. 20231

ASSOCIATE POWER OF ATTORNEY

Sir:

The undersigned attorney has the power of attorney in the subject patent. He hereby grants an associate power to:

Peter C. Lauro, Esq.
Registration No. 32,360
Lahive & Cockfield, LLP
28 State Street
Boston, MA 02109

Please continue to forward all written and telephonic communications to Elizabeth A. Hanley, Esq. at the address and telephone number listed below.

Respectfully submitted,

Giulio A. DeConti, Jr., Esq.
Registration No. 31, 503
Attorney for Applicants

LAHIVE & COCKFIELD, LLP
28 State Street
Boston, MA 02109
Tel. (617) 227-7400

Dated: April 3, 2000